A QUANTITATIVE STUDY, BY MEANS OF SPECTROGRAPHIC ANALYSIS, OF ZINC IN NUTRITION [1]

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ONE FIGURE

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Whether zinc is an essential element in human nutrition is a debated question. Bertrand and Bhattacherjee ('34) reported that mice on a synthetic diet containing less than 0.05 mg. per 100 gm. died in 14 to 23 days, while littermates receiving 2 mg. per 100 gm. lived 57 to 74 days. Newell and McCollum ('33) observed no difference in growth between the rats receiving zinc and those on a zinc-free diet. This lack of difference is attributed by Stirn, Elvehjem and Hart ('35) to incomplete removal of the zinc from the casein used in the ration. Newell and McCollum ('33) state that only the crudest quantitative estimates were attempted by the spectrographic method. They accepted the absence or presence of the 4811 Angstroms line, the one which was present when 0.001 mg. of zinc was present in a 10-gm. sample, as indicating zinc-free diets since it was less than 1 part per million. Using the same spectrographic technic employed by Newell and McCollum ('33), Bayle and Amy ('27) were able to detect even smaller amounts of zinc. This suggests that the zinc content of the test ration used by Newell and McCollum ('33) introduced a supply adequate to meet the requirements of the

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growing animal. Furthermore, Stirn, Elvehjem and Hart ('35) were unable to remove the zinc from milk by the method of electrodialysis, the method employed by Newell and McCollum. They were able, however, to get a zinc-low milk serum product by precipitation with ammonium hydroxide. And, since it was difficult to remove the zinc from the casein, these authors used egg-white with a small amount of casein to provide the protein of the diet. When the resulting zinc-low diet was fed, growth inferior to that obtained with the added zinc was observed.

Hubbell and Mendel ('27) obtained better growth with the addition of 0.02 mg. of zinc to a low zinc diet containing 0.00005 mg. per day than with the zinc-low diet itself. The growth did not, however, equal the growth of the control animals whose diet was an ordinary laboratory ration which was estimated to contain 0.318 mg. of zinc daily. Since the addition of even greater amounts of zinc, 0.04 mg., give less marked growth than the 0.02 mg., the authors concluded that it is possible that the value of zinc lies not alone in the metal itself, but that it may in someway be associated in function with other metals present in small amounts.

The findings of Bertrand and Bhattacherjee ('34) and the later work of Todd, Elvehjem and Hart ('34) confirm the conclusion that zinc is an essential dietary constituent.

That many of the common foods consumed by man are rich in zinc is evident from the published analyses of Fairhall ('26) and Bertrand and Benzon ('28). The former estimated that man ingested about 10 to 15 mg. of zinc per day. Weitzel ('14, '19), Rost and Weitzel ('19) and Rost ('21) found that zinc in human urine ranged from 0.6 to 1.6 mg. per day, with a total elimination from urine and feces of 3.0 to 19.0 mg. Drinker, Fehnel and March ('27) reported the zinc content of the urine of adults as containing from 0.25 to 2.0 mg. per day. The fecal content of these same individuals ranged from 2.67 to 19.9 mg. per day. When foods were selected to increase the zinc content of the diet, urinary excretion did not increase appreciably while the fecal elimination corresponded

more nearly with the intake, frequently extending over a period of several days.

The association of zinc with the metabolism of carbohydrates, which has been reported since this study was undertaken, intensifies the need for zinc metabolism studies. At the time the present study was undertaken it was thought that if zinc is necessary for physiological functioning, it might be determined by means of balance studies with children since during growth zinc should be retained in appreciable amounts. The purpose of this study was to determine how much, if any, zinc is retained by children of preschool age. The zinc lines of the spectrograms of ashed food, feces, urine, distilled water and acid alcohol were compared with spectra produced by solutions of known zinc content.

MATERIALS AND METHODS

The diets employed, the care used in handling and collecting of food and excreta, the preparation of aliquots for spectrographic analysis and the method used in reading the spectrograms is given by Scoular ('38).

Since the wedge spectrograms used in the copper study were also used to obtain the zinc retentions, all precautions which were employed to prevent contamination with copper, also prevented the addition of extraneous zinc.

For the quantitative determination of zinc two standard solutions were prepared to serve as checks. One was made by dissolving a known weight of pure zinc ribbon [3] in hydrochloric acid, and the other by dissolving a known weight of zinc precipitated for the purpose of purification by 8-hydroxyquinoline [4] in hydrochloric acid. Both were made up to volume with redistilled water, so that 1 ml. contained 1.01 mg. of zinc. When the spectrograms were made of the two solutions, the lengths of the zinc lines, 3345 Angstroms, agreed within the reading error of 0.2 mm.

[3] Pure zinc ribbon furnished Waldbauer and Gantz ('33) by the Jersey Zinc Company.

[4] Method described in Organic Reagents for Metals, Staff of Research Laboratory of Hopkins and Williams, Ltd., 1933.

The effect of the sodium chloride, present in the ash of the unknowns, upon the length of the copper line suggested that it might also have some effect upon the zinc lines. Consequently, two sets of electrodes were prepared from the spectrographic quality graphite, both of which contained the same concentration of zinc, but one had in addition to the zinc, 4 drops of a saturated solution of pure sodium chloride. When the spectrograms of these two sets of electrodes were compared, it was found that there was less difference between the zinc line 3345 Angstroms and the reference bands of 3889 Angstroms when the electrode contained sodium chloride. This effect was similar to that observed with copper when sodium chloride was present in the electrode. Therefore, both zinc lines, with and without the addition of sodium chloride, were plotted for a given concentration (fig. 1).

The selection of the zinc line to be read in obtaining the differences between its length and that of the reference bands, 3889 Angstroms, offered no problem with the standard solutions. But in the case of the unknown solutions it was found that the sodium line, 3303 Angstroms, which was present in the spectra of all food, feces and urine samples, masked the zinc lines 3302.9 and 3302.6 Angstroms. The zinc line 2139 Angstroms in the ultraviolet region could not be read because plates sensitized for that region had not been used. Since the triple band at 4680–4722–4810 Angstroms was not clear, reported by Lamb ('35) to be due to the incandescent effect of the graphite after the residue had been burned out, the lines 3345.0–3345.5 Angstroms, which were never separated but considered as 3345 Angstroms in this study, were used in reading the lines of all spectrograms. Some difficulty was experienced in getting clear-cut measurements due to the calcium line 3344.5 Angstroms which was present in some spectra, its concentration being such that it tended to blur the ends of the zinc lines, thus making it difficult to get reading checks within 0.2 mm. To lessen the error when this was encountered, three or more readings were made and the average difference used in comparing concentrations of duplicate samples.

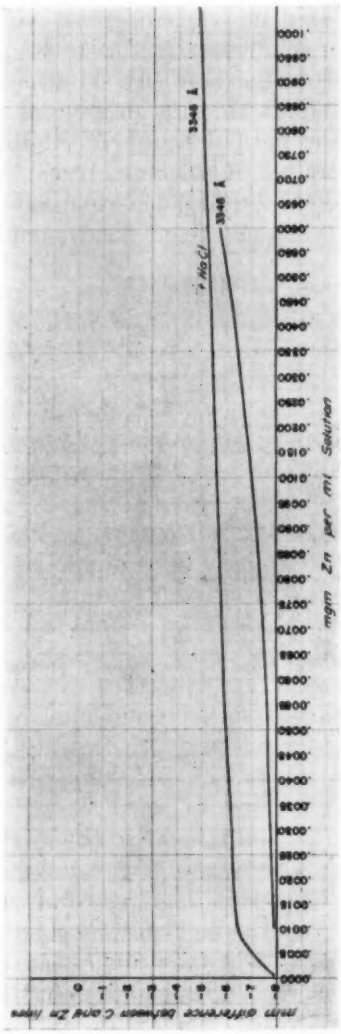

Fig. 1 Difference between the length of the zinc line 3345 Angstroms and the reference bands 3889 Angstroms plotted for each concentration of zinc, with and without added sodium chloride.

The 'best-fit' line, as described by Scoular ('38) in the copper study, was used in determining the concentration of zinc in the unknown solutions. In this way the zinc concentration was read from the line (fig. 1) which most nearly approximated the sodium chloride present in the unknowns. The feces offered the most difficulty in reading, due to the tendency of the quantity of calcium present to overshadow the zinc lines. These technical difficulties are well illustrated by the zinc recovery values from fecal samples which are

TABLE 1

Amount of added zinc recovered from samples of food, feces and urine

PERIODS	SAMPLES TESTED, CONCENTRATION	SAMPLE PLUS 0.005 MG. Zn		
		Concentration	Recovery	
	mg.	*mg.*	*mg.*	%
Urine				
G.E. 2/9	0.0010	0.0060	0.0050	100
F.V. 2/9	0.0005	0.0060	0.0055	110
J.E. 2/9	0.0009	0.0060	0.0051	102
Feces				
G.E. 2/9	0.0400	0.0500	0.0100	200
F.V. 2/9	0.0500	0.0625	0.0125	250
J.E. 2/9	0.0600	0.0725	0.0125	250
	Same samples of feces diluted 1: 10 with redistilled water			
G.E. 2/9	0.0040	0.0085	0.0045	90
F.V. 2/9	0.0060	0.0110	0.0050	100
J.E. 2/9	0.0060	0.0110	0.0050	100
Food				
G.E. 2/9	0.0068	0.0125	0.0057	114
F.V. 2/9	0.0055	0.0100	0.0045	90
J.E. 2/9	0.0055	0.0110	0.0055	110

given in table 1. The addition of 0.005 mg. of zinc to the electrodes containing the fecal ash gave recoveries of from 200 to 250% before these solutions were diluted, and from 90 to 100% after dilution. The high calcium concentration prior to dilution, was believed to be the primary cause of these high figures since the other recoveries had been made with samples containing much less zinc. The difference in the analyzed concentration of zinc in the fecal samples before and after dilution, which varied from 0.000 to +0.001 mg., indicated the

correctness of this assumption. The average range of error for Fairhall and Richardson's ('30) turbidimetric method, which was used by Newell and McCollum ('33) when quantitative determinations were desired, was given as 0.060 mg. Therefore, it was assumed that the concentration of zinc obtained from the readings of the spectra which were made from undiluted solutions of fecal ash was valid and dilution was resorted to only when recoveries were desired.

RESULTS AND DISCUSSION

The spectrograms of distilled water used for drinking purposes showed that the water contained a uniform amount of zinc, namely, 0.001 mg. per 500 ml. The acid-alcohol used on the food and feces, however, contributed from 0.002 to 0.010 mg. per 100 ml., the alcohol of the solution being the source of the zinc. Newell and McCollum ('33) similarly found that alcohol was a source of zinc contamination in one batch of casein in which alcohol was used in purification.

Spectrographic analysis of the diets given in table 2 shows that the ingestion of zinc by the children studied varied from 4 to 6 mg. This variation apparently was not related directly to the changes introduced in the several diets but rather to the varied amounts contained in staple foods. The theoretical intake which was calculated on the basis of the analyses of Fairhall ('26) and Bertrand and Benzon ('28), suggested that diet I (Scoular, '38) contained less zinc than diet II, and that the latter was not as rich as diet III. All of the estimated values, however, were from 3.5 to 3.6 mg. higher than those obtained by spectrographic analysis. The average difference between the zinc content of the three diets as estimated and as analyzed, were almost identical, being 1.3 mg. for the spectrographic method and 1.4 for the theoretical estimate. The close relationship to these values suggest that the differences are due to methods. In the present study the highest ingestion level was only 41% higher than the lowest ingestion. From an inspection of table 2, the differences in zinc composition of the individual diets, within this limited range of

TABLE 2

Average daily intake, excretion and retention of zinc

DIET NO.	CASE DATE	ZINC INGESTION					ZINC EXCRETION				RETENTION	
		Food		A.A.	Total		Feces	Urine	A.A.	Total	Total	Per kg.
		Theoretical[1]	Analyzed		Mg.	Per kg.						
		mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
II	F.V. 3/22	7.79	4.48	0.01	4.47	0.218	3.44	0.01	0.01	3.46	1.01	0.045
I	J.E. 2/9	7.83	4.29	0.01[2]	4.28	0.222	4.66	0.02	0.00[2]	4.68	—0.40	—0.019
I	F.V. 2/9	7.57	4.22	0.01[2]	4.21	0.222	4.33	0.01	0.00[2]	4.34	—0.13	—0.005
II	G.E. 3/22	7.46	3.81	0.01	3.80	0.223	6.40	0.10	0.00	6.50	—2.70	—0.159
III	F.V. 6/7	8.60	4.91	0.01	4.90	0.223	3.43	0.16	0.00	3.59	1.31	0.063
III	F.V. 5/12	8.60	4.58	0.01	4.57	0.224	4.46	0.21	0.00	4.67	—0.10	—0.005
I	J.E. 2/4	7.83	4.37	0.01[2]	4.36	0.226	4.07	0.14	0.00[2]	4.21	0.15	0.008
I	F.V. 2/4	7.57	4.32	0.01[2]	4.31	0.227	4.27	0.03	0.00[2]	4.30	0.01	0.001
III	F.E. 5/17	8.60	4.68	0.01	4.67	0.227	2.27	0.28	0.00	2.55	2.12	0.112
II	G.E. 3/18	7.46	3.89	0.01	3.88	0.229	2.41	0.01	0.00	2.42	1.46	0.082
II	J.E. 3/22	7.99	4.56	0.01	4.55	0.230	3.09	0.17	0.00	3.26	1.29	0.069
III	J.E. 5/12	8.67	4.79	0.01	4.78	0.234	4.30	0.09	0.00	4.39	0.39	0.019
III	J.E. 4/21	8.64	4.79	0.01	4.78	0.237	2.02	0.02	0.00	2.04	2.74	0.135
II	G.E. 3/1	7.46	4.04	0.02	4.02	0.239	6.62	0.01	0.01	6.64	—2.62	—0.131
III	F.V. 6/2	8.60	5.01	0.01	5.00	0.240	3.74	0.07	0.00	3.81	1.19	0.056
III	J.E. 5/17	8.67	4.95	0.01	4.94	0.241	5.44	0.13	0.00	5.57	—0.63	—0.030
III	J.E. 4/26	8.64	4.88	0.01	4.87	0.241	4.72	0.04	0.00	4.76	0.11	0.011
III	J.E. 6/7	8.67	5.09	0.01	5.08	0.246	4.37	0.00	0.00	4.37	0.71	2.033
III	F.V. 4/26	8.58	5.51	0.01	5.00	0.246	2.90	0.01	0.00	2.91	2.09	0.101
II	F.V. 3/1	7.79	4.85	0.02	4.83	0.249	6.52	0.01	0.01	6.54	—1.71	—0.091
III	G.E. 4/26	7.48	4.29	0.01	4.28	0.250	5.08	0.09	0.00	5.17	—0.89	—0.023
II	J.E. 3/17	7.99	4.94	0.01	4.93	0.250	4.61	0.16	0.00	4.77	0.16	0.010
III	J.E. 6/2	8.67	5.19	0.01	5.18	0.251	3.70	0.16	0.00	3.86	1.32	0.064
III	F.V. 4/21[3]	8.58	5.15	0.01	5.14	0.254	3.44	0.01	0.00	3.45	1.69	0.084
III	G.E. 6/7	7.50	4.61	0.01	4.60	0.258	4.93	0.25	0.00	5.18	—0.58	—0.033
II	F.V. 3/19	7.79	5.05	0.01	5.04	0.259	3.99	0.01	0.00	4.01	1.03	0.050
III	G.E. 5/12	7.50	4.58	0.01	4.57	0.262	4.29	0.12	0.00	4.41	0.16	0.004
III	G.E. 4/21	7.48	4.56	0.01	4.55	0.264	4.37	0.01	0.00	4.38	0.17	0.010
II	J.E. 3/1	7.93	5.08	0.02	5.06	0.266	6.27	0.01	0.01	6.29	—1.23	—0.063
III	G.E. 5/17	7.50	4.74	0.01	4.73	0.269	4.50	0.18	0.00	4.68	0.05	0.004
I	G.E. 2/9	7.31	4.65	0.01[2]	4.64	0.279	3.38	0.00	0.00[2]	3.38	1.26	0.075
III	G.E. 6/2	7.50	5.03	0.01	5.02	0.282	3.38	0.01	0.00	3.39	1.63	0.094
I	G.E. 2/4	7.31	4.79	0.01[2]	4.78	0.288	3.10	0.02	0.00[2]	3.12	1.66	0.100
II	F.V. 2/25	7.79	5.88	0.01	5.87	0.300	2.47	0.00	0.01	2.48	3.39	0.171
II	G.E. 2/25	7.46	5.21	0.01	5.20	0.307	3.11	0.01	0.01	3.13	2.07	0.123

[1] Estimated from values published by Fairhall ('26) and Bertrand and Benson ('28).

[2] Estimated values.

[3] Slight regurgitation which was not analyzed.

zinc ingestion, appear more significant than the actual differences warrant. For example, when the difference in the ingestion level of F.V. and J.E., two children of about the same age and weight, for period 3/22 is calculated, J.E.'s is higher by only 5%. However, when tabulated in the order of increasing zinc ingestion levels, nine cases fill the intervening gap of 5%. Since the range of error estimated for the spectrographic method used in this study approximated 10%, this difference in the zinc content of the diet for period 3/22 loses its significance. The determined zinc values of the foods used in two consecutive 5-day metabolism periods were very close, all falling within the 10% range of error.

The amount of zinc excreted in the urine was small, representing from 0.04 to 6.0% of the intake. This was not related to intake nor was it constant for a given individual. In some cases there was little change from period to period, in other instances there were wide variations.

Zinc is obviously excreted through the alimentary tract. The fecal output ranged from 2.016 to 6.624 mg. per day, representing from 42 to 163% of the ingested zinc. At times there was almost no difference between the amounts of zinc excreted in the feces of the two 5-day metabolism periods as in 2/4 and 2/9, and 3/19 and 3/22 for F.V. On the other hand, periods 5/12 and 5/17, and 2/25 and 3/1 for the same child (F.V.) varied more than 50%. These excretions bore no relation to the differences noted in the urinary excretion, nor to the amount of zinc ingested, although there was a tendency toward higher fecal zinc with the higher ingestion levels. These findings corroborate those of Drinker, Fehnel and March ('27) that urinary excretions of zinc did not increase appreciably with high zinc intake, while the fecal elimination corresponded more nearly with the intake, frequently extending over a period of several days. Some of the negative balances in the present study may have resulted from delayed excretion.

Of the thirty-five balances tabulated (table 2), ten are negative with a range of —0.005 to —0.131 mg. per kilogram of body weight. And six of these ten occurred with an ingestion

[1] Estimated from values published by Fairhall ('26) and Bertrand and Benzon ('28).
[2] Slight regurgitation which was not analyzed.
[3] Estimated value.

level which was lower than the average (0.248 mg. per kilogram of weight) for the entire study. Of the twenty-five positive balances, the highest retention (0.171 mg.) occurred when F.V. received his highest ingestion level, namely, 0.300 mg. per kilogram of body weight. However, J.E. showed his highest retention of 0.135 mg., with an ingestion level below that of the average, namely, 0.237 mg. per kilogram of body weight. Since the two highest retentions, not including the exception just mentioned, were obtained with the two highest ingestion levels, 0.300 and 0.307 mg. per kilogram, it would seem that higher ingestion levels might give better retentions. On this basis it might be tentatively concluded that an ingestion of not less than 0.300 to 0.307 mg. of zinc per kilogram of body weight is necessary for the preschool age child.

SUMMARY

Thirty-five zinc balance studies have been made with three normal boys of preschool age.

The ashed food, feces, urine, distilled water and acid-alcohol were analyzed spectrographically for zinc by comparing their spectra with those produced by solutions of known concentration.

The zinc values for food which were analyzed by quantitative spectrographic technic were found to be almost 40% lower than the theoretical values estimated from published analyses for which the turbidimetric method had been used.

From 0.04 to 6.0% of the ingested zinc was eliminated through the urine. The rest of the excreted zinc, representing from 42 to 164% of the ingested amount, was eliminated through the alimentary tract.

Since more than two-thirds of the thirty-five balance studies gave retentions which were significantly greater than the error determined for the method used, it would seem that zinc is associated with physiological functioning.

On the basis of the limited ingestion range studied it is tentatively concluded that 0.307 mg. of zinc per kilogram of body weight will supply the zinc needs of the preschool age child.

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LITERATURE CITED

BAYLE, E., AND L. AMY 1927 Application of spectrographic analysis to the estimation of metallic impurities. Compt. rend. Acad. Sci., T. 185, p. 268.

BERTRAND, G., AND B. BENZON 1928 Zinc content of some plants. Compt. rend. Acad. agr. France, T. 14, p. 1303.

BERTRAND, G., AND R. C. BHATTACHERJEE 1934 Combined action of zinc and vitamins in animal nutrition. Compt. rend., T. 198, p. 1823.

DRINKER, K. R., J. E. FEHNEL AND M. MARCH 1927 Normal excretion of zinc in the urine and feces of man. J. Biol. Chem., vol. 72, p. 375.

FAIRHALL, L. T. 1926 Estimation of zinc in biological materials. J. Ind. Hyg., vol. 8, p. 165.

FAIRHALL, L. T., AND J. R. RICHARDSON 1930 Nephelometric analysis of zinc. J. Am. Chem. Soc., vol. 52, p. 938

HARRISON, G. H. 1936 Practical possibilities in spectrographic analysis. Metals and Alloys, vol. 7, p. 291.

HUBBELL, R. B., AND L. B. MENDEL 1927 Zinc and normal nutrition. J. Biol. Chem., vol. 75, p. 567.

LAMB, F. W. 1935 Spectrographic determination of impurities in commercial cadmium. Proc. Am. Soc. Testing Materials, Part II, vol. 35, p. 71.

NEWELL, J. M., AND E. V. MCCOLLUM 1933 Studies on the role of zinc in nutrition. J. Nutrition, vol. 6, p. 289.

ROST, E. 1921 Woher stammt das Zink in menschlichen und tierischen Organismus. Med. Klin., Bd. 17, S. 123.

ROST, E., AND A. WEITZEL 1919 Zur Kenntnis des Vorkommens von Zink (und Kupfor) in den Ausscheidungen und Organen des Menschen und in unser Lebensmitteln. Arb. Reichsgsndhtsamte, Bd. 51, S. 494.

SCOULAR, FLORENCE I. 1938 A quantitative study by means of spectrographic analysis of copper in nutrition. J. Nutrition, vol. 16, p. 437.

STIRN, F. E., C. A. ELVEHJEM AND E. B. HART 1935 Indispensability of zinc in nutrition of the rat. J. Biol. Chem., vol. 109, p. 347.

TODD, E. R., C. A. ELVEHJEM AND E. B. HART 1934 Zinc in the nutrition of the rat. Am. J. Physiol., vol. 107, p. 146.

WEITZEL, A. 1914 Über das naturliche Vorkommen von Zink in Lebensmitteln und in the Ausscheidungen of Menschen. Zentr. Physiol., Bd. 28, S. 766.

———— 1919 Beitrage zur Bestimmung von Zink in organischen Stoffen—Harn, Kot, Lebensmitteln usw.—nebst Bemerkungen über den Zinkgehalt von Reagentien und Analysengefassen. Arb. Reichsgsndhtsamte, Bd. 51, S. 476.

RAT ACRODYNIA AND THE ESSENTIAL FATTY ACIDS [1]

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The first dietary studies which suggested a relationship between dermal abnormalities and fat deficiency were made by Burr and Burr ('29, '30). On their diet which contained ether-extracted yeast, rats developed mild skin lesions, consisting of scaliness of paws and tail, in 8 to 10 weeks. The symptoms were healed by the administration of unsaturated fats, or specifically, esters of linoleic acid. Hogan and Richardson ('34), and Richardson and Hogan ('36), using a lower level of yeast which had been irradiated, obtained severe symptoms in 5 to 6 weeks. The use of either cornstarch or rice starch in the diet prevented their occurrence; butterfat likewise delayed or prevented them. Cures were produced with fats, especially wheat germ oil, but since the activity of the fats did not parallel their unsaturation, the curative properties were not believed to be due to a fatty acid. Cures were also obtained with aqueous solutions and their fuller's earth adsorbates. György ('35) produced dermatitis, later called rat-acrodynia, with fair success on a diet consisting chiefly of rice starch and containing 9% butterfat. The symptoms did not appear until the seventh to the eighth week, however, and in some cases disappeared spontaneously.

[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station, Madison, Wisconsin.

The most potent curative materials tested were Peter's eluate, equivalent to 10 gm. of baker's yeast daily, and wheat germ. While Birch and György ('36) had difficulty in getting the entire activity into aqueous solution, they were able to prepare concentrates by adsorption on fuller's earth and by electrodialysis. Cures with lard or linseed oil were also reported. Halliday and Evans ('37), using sucrose and only 3% butterfat in the diet, reported the production of a dermatitis within 3 to 4 weeks. Lepkovsky, Jukes and Krause ('36) using an almost identical diet except that it contained 10% lard did not obtain dermatitis unless they added a 'filtrate factor,' factor 2. Cures were obtained with a fuller's earth adsorbate, factor 1. Bender, Ansbacher, Flanigan and Supplee ('36), and Bender and Supplee ('37) found the production of dermatitis irregular on a diet containing 10% hydrogenated cottonseed oil,[2] but upon decreasing the fat to 3% obtained symptoms consistently. Salmon ('38) reported that oils alone or starch alone failed to cure or prevent acrodynia and that oils did not contain the entire dermatitis-preventing factor. Nor did the methyl esters of linoleic and linolenic delay the onset of symptoms. Birch ('38) more recently reported that two factors were concerned: one, water soluble, viz., vitamin B_6 and one fat soluble similar to the fatty acid factor of Burr and Burr. It is evident from the foregoing that the relation of certain constituents of the diet to the production and cure of dermatitis is not revealed clearly. The following results with rats on various diets and fat supplements already reported briefly (Quackenbush and Steenbock, '38) are therefore presented in detail.

EXPERIMENTAL

Diets and the production of dermatitis

The various constituents of the diets are given in table 1. Casein 30 was prepared from commercial casein by extracting it with N/50 acetic acid for 1 week. It was washed free from acid, dried at 50°C. and ground, then extracted twice with

[2] Crisco.

1.5 liters of alcohol per kilo at 45 to 50°C. for two periods of 2 days each, filtered on a large Büchner funnel and again dried.

The irradiated yeast was prepared from dried brewer's yeast (Anheuser-Busch). It was ground to pass a 100-mesh sieve, and irradiated with occasional stirring in thin layers, 400 gm. per square meter, with a large cold quartz burner at 16 cm. The temperature of the yeast did not rise above 30°C. One sample was irradiated for 5 hours; another for 25 hours. One hundred milligrams of these were fed as daily supplements for diets I and II respectively.

TABLE 1
Primary constituents of diets[1]

DIETS	CASEIN 30	WESSON SALTS	CARBOHYDRATE		OILS	
	gm.	*gm.*	*gm.*		*gm.*	
VI	18	4	Cerelose	68	Hydrogenated coconut	10
VII	18	4	Cerelose	68	Peanut	10
VIII	18	4	Cerelose	68	Wheat germ	10
V	18	4	Cerelose	78		
XII	18	4	Sucrose	78		
XIII	18	4	Rice starch	78		
I	20	4	Sucrose	71	Cod liver	2
II	20	4	Sucrose	71	Cod liver	2

[1] For supplements see text.

The mineral elements were supplied as Wesson salts (Wesson, '32) or salts P, composed of NaCl 5.0, $CaCO_3$ 36.0, $CaCl_2.6H_2O$ 28.4, $CaHPO_4.2H_2O$ 21.8, $FeCl_3.6H_2O$ 2.4. Agar was fed at a level of 3% in diets I and II. Daily vitamin supplements consisted of 10 micrograms B_1 (Merck's synthetic), 20 micrograms riboflavin, 10 micrograms β-carotene, and 5 micrograms calciferol, except for diets I and II. The B_1 and riboflavin were dissolved in N/50 acetic acid and fed from a medicine dropper. In all crucial experiments synthetic riboflavin (Hoffman-La Roche) was used. In routine assays, however, either a concentrate of high purity or the natural crystalline compound was used. Liquid hydrogenated coconut oil was used for the administration of calciferol and carotene and for the dilution of the more potent oils. It was

prepared by crystallizing out the solid fats from a coconut oil which had been hydrogenated to an iodine number of less than 0.20. Tests showed it to be biologically inactive. One drop of the solution was given to each rat daily.

All of the animals which received either no fat or hydrogenated coconut oil developed well-defined symptoms in 8 to 10 weeks. With the addition of 10% peanut oil or wheat germ oil to the diet lesions were not produced. Since our stock diet (Steenbock, '23) consisted chiefly of natural grains, it was

TABLE 2

Incidence of dermatitis on the various diets

DIET	NUMBER OF ANIMALS	DIET DURING NURSING PERIOD	NUMBER SHOWING MILD SYMPTOMS	NUMBER SHOWING SEVERE SYMPTOMS	AVERAGE TIME FOR SEVERE SYMPTOMS TO DEVELOP	LENGTH OF EXPERIMENT
					weeks	*weeks*
I	7	S	4	0		9
I	5	S	5	0		22
II	11	S	8	0		9
V	17	P		17	4½	5
VI	2	S		2	10	10
VI	5	P	1	4	4½	6
VII	7	P	0	0		7
VIII	2	P	0	0		10
XII	8	P		8	6	14
XIII	9	P	5	4	8+	14

S, stock diet. P, potato diet.

assumed that initial storage in the experimental animals might account for the slow rate at which dermatitis was produced. Departing from the use of grains, diet P composed of potato meal 90 gm., salts P, 1.6 gm., casein 30, 8.4 gm. was fed. The mother rat received 22 mg. halibut liver oil twice a week during the nursing period. The potatoes were steamed for 40 to 45 minutes, sliced and dried on wire screens before a fan at room temperature and then ground to a meal. Each daily portion of the diet was moistened before feeding.

On diet V the rats developed an acute dermatitis in 4 to 5 weeks (table 2). When sucrose was used instead of cerelose the symptoms were retarded slightly. With rice starch, on

the other hand, less than half of the animals developed a severe dermatitis within 14 weeks. By this time most of the animals were quite emaciated. Neither Labco nor Glaxo casein substituted for casein 30 hastened the appearance of symptoms. When irradiated yeast supplemented diets I and II similar to Hogan and Richardson ('34) diet only mild symptoms of dermatitis were produced (table 2).

The following standard technic was adopted for the production of animals for curative studies: females with litters of four to six young were transferred within 2 days after delivery, from the stock diet to diet P. Ten days later they were placed on screens. When the young reached a weight of 40 gm., usually during the fourth week, they were weaned and given diet V. After 5 weeks on this diet dermatitis developed without exception. Spontaneous recoveries were not observed.

Two well-defined symptom complexes, the acute and the chronic form, were recognized. In the acute form the lips, eyes, and fore paws were the areas chiefly involved. The animals became inactive and lost from 5 to 10 gm. in weight. Without supplement, most of the animals died within a few days.

The chronic form usually developed in animals which received supplements of low potency. The lesions were very extensive. The entire peripherae, especially the ears and hind paws became involved. The lips and fore paws were generally parchmentized. Ringlets of scales appeared on the tail which sometimes sloughed off leaving a shiny, denuded surface. The torso was usually not denuded at all, but in the chronic form it was covered with minute adherent scales. The chronic form could not be differentiated from György's 'rat-acrodynia.'

The initial nature of the dermatitis and the length of the required assay period were important factors in the determination of potencies. To evaluate them properly the symptoms were charted systematically (table 3).

Within 2 to 3 weeks after the administration of a potent supplement, the swelling and inflammation disappeared, the skin was regenerated and the hair was restored in all the areas involved. The lips and eyes healed more rapidly than the paws. The last visible symptom to disappear was a dry scaliness of the hind paws and tail, and occasionally of the

TABLE 3

Stages of dermatitis

	STAGE 1	STAGE 2	STAGE 3	STAGE 4
Lips	Hyperemia, usually with small ulcers at corners	Slight swelling, redness along vertical groove	Medium swelling, desquamation to nostrils	Severe swelling, raw soreness of wide area around mouth and nostril
Eyes	Lids wet, slight crusts at the corners	Slight swelling of lids, crusts in corners	Medium swelling, lids often adherent with gummy exudate	Severe swelling, lids swollen shut, large crusts on lids
Fore paws	Slight scaliness, redness following desquamation between digits	Slight swelling digits desquamated, red and sore	Medium swelling, digits raw and sore, sometimes with pus accumulation	Severe swelling, desquamated and sore to wrists; gangrenous
Hind paws	Same as fore paws	Same as fore paws; also desquamation of large areas on paws	Same as fore paws; skin cracked and ulcerated at heel and along tibia	Severe swelling; large red areas over paws and inner thigh, paws often gangrenous
Ears	Hyperemia, slight swelling	Pinnae swollen and thickened due to scale formation	Medium thickening at lips, large scales sloughing	Severe thickening, necrosis
Tail	Scaly (ringlets)	Very scaly	Very scaly; necrosis near tip	

fore paws. In some cases a condition of scaliness persisted for several weeks, even when the amount of supplement had been increased tenfold. This was especially noticeable with yeast supplements; and it was most persistent during the winter months, probably because of the lower humidity (Brown and Burr, '36). Accordingly, absence of scaliness was not used as a criterion of potency.

The method used for quantitative assays was standardized as follows: animals were given supplements as soon as the lips and paws reached stage 2 to 3 (table 3). A cure was assumed to have been effected if at the end of a 21-day period of assay the lips, eyes and ears were normal in appearance and the paws and tail free from all symptoms except a dry scaliness. A minimum curative dose was defined as that amount which when given daily produced cures in more than 50% of the animals, with none failing to respond to twice the amount of the same supplement.

TABLE 4

Potencies of oils

OIL SAMPLE	MINIMUM CURING	MAXIMUM FAILING
	drops/day	*drops/day*
Wheat germ (commercial)	½	¼
Wheat germ (ether extract)	½	...
Cottonseed (Wesson)	½	...
Corn (Mazola)	½	...
Peanut (commercial)	1	½
Peanut (ether extract)	1	...
Linseed (commercial)	2	1
Linseed (ether extract)	2	1
Lard	2	...
Olive	5	2
Butterfat	25	10
Coconut	10	5
Hydrogenated coconut	...	10
Cod liver	...	2
Halibut liver	...	½

The curative action of fats

With the standardized technic the minimum curative dose for wheat germ oil, peanut oil, cottonseed oil and corn oil was approximately 0.5 to 1 drop daily. Coconut oil and butterfat were far less potent, requiring 10 to 25 drops daily. Hydrogenated coconut oil was entirely inactive (table 4). It might be inferred from these results that the biological activity paralleled the degree of unsaturation. However, the results with cod liver oil and linseed oil did not support this inference;

they were both less active than the more saturated wheat germ, peanut, cottonseed and corn oils.

The permanency of the cures obtained was determined with twenty animals which had been used in routine assays. All had developed severe dermatitis in 4 to 5 weeks on diet V, and all had been cured with fat supplements by the end of the eighth week. Five of these animals were given diet V without supplement; within 4 to 5 weeks all had again developed the acute symptoms in their original severity. The other fifteen animals continued to receive small supplements of peanut oil, wheat germ oil, lard or butterfat, and at the end of the same period showed—except for an occasional scaly tail—none of the symptoms. Three of the animals which received diet V which had been supplemented with 3% peanut oil were continued on the diet for 27 weeks, without recurrence of the symptoms.

The effect of various treatments on curative properties

Oxidation. A sample of peanut oil having a peroxide number of 15 was placed in an aspiration cell placed directly below a 60-watt Mazda lamp. Air was bubbled through the oil at a temperature of 50 to 60°C. for a period of 72 hours. Although the peroxide number was increased to 34, the oil was still curative when fed at 2 drops per day.

Irradiation. A sample of peanut oil was placed under nitrogen in a quartz container and irradiated at a distance of 15 cm. with a cold quartz lamp for 10 hours. The irradiated oil produced cures when 2 drops were fed daily.

Saponification. A sample of peanut oil was saponified with KOH in alcohol under nitrogen. The reaction mixture was extracted thoroughly with ether, and the extract evaporated to dryness. The residue was diluted with hydrogenated coconut oil so that 1 drop of the mixture contained the unsaponifiable fraction from 5 gm. of peanut oil. This material failed to cure dermatitis even when 2 drops were fed daily.

The ether-extracted soaps were acidified with HCl and the fatty acids taken up with ether. After washing and drying the solution, the ether was removed under reduced pressure.

When the fatty acids were fed at a level of 3 drops per day, the animals developed severe localized irritation and failed rapidly. A portion of the fatty acids was esterified by refluxing with absolute alcohol in the presence of H_2SO_4. The ethyl esters were washed thoroughly with water and dried by warming under reduced pressure. The product was fed at 3 drops per day to animals in both the acute and chronic forms. Complete cures were obtained in all cases.

A sample of wheat germ oil was saponified and the mixed ethyl esters were prepared by the method described above. The product was found to have the same potency as the original oil. It was used for fractionations as described below. The acids from corn oil were also fractionated.

Fractional distillation of the ethyl esters from wheat germ oil

Distillates from 40 gm. of the ethyl esters prepared from wheat germ oil were collected at 1 mm. pressure at 150 to 200°C. Of the various fractions only two, namely, those distilling at 170 to 173°C. (D_2) and at 173°C. (D_3) were found to be biologically potent when given in amounts of 1 drop per rat daily. D_2 (170 to 173°C.) was the only fraction which gave some improvement at $\frac{1}{10}$ drop. These two fractions comprised 60% of the total distillate. No further concentration of potency was obtained by their redistillation.

Fractional crystallization of fatty acids from wheat germ oil

The fatty acids were crystallized from acetone according to the method of Brown and Stoner ('37). The fractions crystallizing above —25°C. were found to be biologically inert; the first potency was obtained with the fraction crystallizing at —45°; those separating between —50 and —75° showed the highest potency (table 5).

Preparation of ethyl linoleates from corn oil

Three samples of ethyl linoleate were prepared from the fatty acids of corn oil. The first was obtained from fatty

acids which had been fractionally crystallized from acetone according to the method of Brown and Stoner ('37). The second was prepared from fatty acids which had been separated by bromination according to Rollett ('09). The third was obtained by the same process as the second followed by elaidinization with nitrous acid and recrystallization of the product from acetone.

Properties of the esters

The three ester preparations were colorless oils at room temperature, and all solidified upon cooling to −60°C. The ethyl α-linoleate and the ester obtained by direct fractional

TABLE 5

Potency of the fatty acids of wheat germ oil crystallized from acetone

FRACTION	CRYSTALLIZATION TEMPERATURE	YIELD	I.V. OF ETHYL ESTERS HANUS	MINIMUM CURING	MAXIMUM FAILING
	°C.	gm.		drops/day	drops/day
C_1	0	4.0	10	..	1
C_2	−25	25.3	70	..	1
C_3	−45	17.8	117	1	½
C_4	−50	20.2	141	½	1/10
C_5	−55	12.5	149	½	1/10
C_6	−75	8.2	153	½	1/10
C_7	Filtrate	3.2	165	1	½

crystallization melted at −55°; the elaidinized product at −18°. The iodine values (Hanus) were 165, 158, 160, respectively. The data for these esters supply further evidence for the hypothesis of Brown and Frankel ('38) that linoleic acid prepared by direct fractional crystallization is identical with α-linoleic acid.

When the esters were assayed for biological activity, the ethyl linoleates obtained from linoleic acid by direct crystallization and as the hexabromide were active in doses of ½ drop daily, the elaidinized ester was found to be inactive at 1 drop daily. All were inactive when given in amounts of 1/10 and ½ drop per rat daily.

SUMMARY

On a basal diet of purified casein, glucose and salts, supplemented with carotene, calciferol, synthetic B_1 and riboflavin, rats developed skin lesions in 4 to 5 weeks; an acute dermatitis when restricted to the basal diet; and a chronic form, 'rat-acrodynia,' when given fat supplements at subcurative levels.

Complete healing of either the acute or chronic form was obtained by the administration of peanut oil or wheat germ oil. Cured animals maintained themselves free from all symptoms so long as the fat supplements were continued or until the end of the survival period, which was usually several months. Aeration or ultraviolet radiations did not destroy the activity in peanut oil. It was resident in the fatty acid fraction.

Cures were produced with ½ drop of wheat germ oil, corn oil or Wesson oil, 10 drops of coconut oil or 25 drops of butterfat. The lesions were not prevented nor cured with hydrogenated coconut oil.

The unsaponifiable fraction from wheat germ oil was devoid of potency; the ethyl esters prepared from the soap fraction contained all of the activity. When the unsaturated fatty acids were fractionally crystallized from acetone, the highest potency was obtained with the fraction separating between −50 and −75°C. One-half drop of ethyl linoleate (Rollett, '09) per day cured the dermatitis completely. The ethyl ester of elaidinized linoleic acid was found to be inactive.

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LITERATURE CITED

BENDER, R. C., S. ANSBACHER, G. E. FLANIGAN AND G. E. SUPPLEE 1936 The influence of dextrin and sucrose on growth and dermatitis. J. Nutrition, vol. 11, p. 391.

BENDER, R. C., AND G. E. SUPPLEE 1937 Significance of quantitative relationships in vitamin B complex studies. J. Am. Chem. Soc., vol. 59, p. 1178.

BIRCH, T. W. 1938 The relation between vitamin B_2 and the unsaturated fatty acid factor. J. Biol. Chem., vol. 124, p. 775.

BIRCH, T. W., AND P. GYÖRGY 1936 A study of the chemical nature of vitamin B_2 and methods for its preparation in a concentrated state. Biochem. J., vol. 30, p. 304.

BROWN, J. B., AND J. FRANKEL 1938 Studies on the chemistry of the fatty acids. J. Am. Chem. Soc., vol. 60, p. 54.

BROWN, J. B., AND G. G. STONER 1937 Studies on the chemistry of the fatty acids. J. Am. Chem. Soc., vol. 59, p. 3.

BROWN, W. R., AND G. O. BURR 1936 Some recent studies on fat deficiency. J. Biol. Chem., vol. 114, p. XVI.

BURR, G. O., AND M. M. BURR 1929 A new deficiency disease produced by the rigid exclusion of fat from the diet. J. Biol. Chem., vol. 82, p. 345.

———— 1930 On the nature and role of the fatty acids essential in nutrition. J. Biol. Chem., vol. 86, p. 587.

GYÖRGY, PAUL 1935 Investigations on the vitamin B_2 complex. I. The differentiation of lactoflavin and the 'rat antipellagra' factor. Biochem. J., vol. 29, p. 741.

HALLIDAY, N., AND H. M. EVANS 1937 On the fractionation of the vitamin B_1 complex from various source materials. J. Biol. Chem., vol. 118, p. 255.

HOGAN, A. G., AND L. R. RICHARDSON 1934 Irradiated vitamin B complex and dermatitis. J. Nutrition, vol. 8, p. 385.

LEPKOVSKY, S., T. H. JUKES AND M. E. KRAUSE 1936 The multiple nature of the third factor of the vitamin B complex. J. Biol. Chem., vol. 115, p. 557.

QUACKENBUSH, F. W., AND H. STEENBOCK 1938 Acrodynia and the essential fatty acids. J. Biol. Chem., vol. 123, p. xcvii.

RICHARDSON, L. R., AND A. G. HOGAN 1936 Skin lesions of the rat associated with the vitamin B complex. Mo. Agr. Exp. Sta. Res. Bull. 241.

ROLLETT, A. 1909 Zur kenntnis der Linolsäure. Z. Physiol. Chem., vol. 62, p. 410.

SALMON, W. D. 1938 The effect of certain oils in alleviating localized erythematous dermatitis (acrodynia or vitamin B_2 deficiency) in rats. J. Biol. Chem., vol. 123, p. civ.

STEENBOCK, H. 1923 A satisfactory ration for stock rats. Science, vol. 58, p. 449.

WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, vol. 75, p. 339.

STUDIES OF FAT-FREE DIETS

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Evans and Burr ('27, '27 a) demonstrated the existence of a deficiency syndrome in the rat produced by the exclusion of fat from the diet. The syndrome was characterized by subnormal weight and poor reproductive performance. Subsequent work by Burr and co-workers ('29, '32) showed that certain fatty acids, linoleic and linolenic, were curative of and prophylactic for this deficiency disease. Turpeinen ('37, '38) has recently suggested that the animal organism might primarily require arachidonic acid rather than linoleic or linolenic acid. His suggestion was based on the demonstration that arachidonic acid was curative at a level of 33 mg., whereas 100 mg. of methyl linoleate was necessary to produce an equivalent optimal growth in plateaued fat-deficient animals. Burr, Burr and Miller ('32) had stated that the maximum growth effect of linoleic acid was reached at a 10-drop level per rat per day; however, Bull ('37) quotes Burr to the effect that ½ drop of linoleate daily is a dosage sufficient to cure scaliness of feet, tail and skin, and to restore growth to a fat-deficient rat. This paper deals with controversial points in the study of fat-free diets.

SECTION I. FAT DEFICIENCY SYNDROME

Procedure

The basal fat-free diet used in these experiments had the following composition:

	%
Casein (alcohol- and ether-extracted)	20.0
Northwestern dried yeast (ether-extracted)	10.0
Sucrose	64.0
Salt mixture no. 51	6.0

Salt mixture no. 51

$CaCO_3$	1.5
KCl	1.0
NaCl	0.5
$NaHCO_3$	0.7
MgO	0.2
Fe citrate	0.5
KH_2PO_4	1.7
	6.1

The ether extractions of casein and yeast were made in Soxhlet apparatus for 24-hour periods. The casein had previously been extracted by first cold 70% and then cold 95% alcohol for a like period of time. Ether extraction over a period beyond 24 hours proved valueless as no additional lipoid material was removed.

One group of animals, seventy in number, was maintained on this basal fat-free diet from weaning with the following supplements:

Carotene[1] (dissolved in ether)	0.08 mg. per rat per day
Ergosterol[1] (irradiated) (dissolved in ether)	100 international units per rat per day
Vitamin E (non-saponifiable matter of wheat germ oil)	≏ 3 gm. wheat germ oil per week

This constituted diet A.

A second group received 10 drops of methyl linoleate from weaning time, the purpose being to compare the growth response made by rats on this diet when linoleate was used prophylactically. Twenty-two rats were placed on this diet.

[1] Supplied through the courtesy of Dr. Charles E. Bills, Mead Johnson and Company.

The supplements were as follows:

Carotene (dissolved in ethyl laurate)	0.08 mg. per rat per day
Ergosterol (irradiated; dissolved in ethyl oleate)	100 international units per rat per day
Vitamin E (non-saponifiable matter of wheat germ oil)	≏ 3 gm. wheat germ oil per week
Methyl linoleate	10 drops or 300 mg. per rat per day

This group of supplements, plus the basal fat-free diet, constitutes diet B.

Methyl linoleate was prepared by esterifying a commercial product of linoleic acid. This product was secured from the Glyco Company and is prepared from linseed oil. The esterification was the methyl alcohol-anhydrous hydrogen chloride technic. Fractionation in a 6-foot, high vacuum, laboratory fractionating column gave a product of iodine number 150.3. The preparation contains both methyl linolenate and methyl oleate, and is referred to as crude methyl linoleate.

For the more refined feeding tests reported herein, a methyl linoleate was prepared by the bromination procedure of Rollet ('09). The methyl linolenate used was also prepared by the method of Rollet ('09 a).

Iodine numbers	*Observed*	*Theoretical*
Crude linoleate	150.3	
Pure linoleate	173.1	181.2
Pure linolenate	259.3	273.7

Even on this diet containing every known essential, the full adult size is not attained. After the growth curves have flattened for several weeks the rats again grow promptly when given our stock diet. Stimulation of a new growth increment was the principal criterion used in the study of certain additions to the diet.

RESULTS

Symptomatology

Burr and Burr ('29) reported caudal necrosis as the most marked symptom in their fat deficiency disease. Scaly feet, dandruff, alopecia and hematuria were listed as other criteria

of the existence of an unsaturated fatty acid deficiency. A year later, Burr and Burr ('30) stated that caudal necrosis does not occur in the first 4 to 6 months on the diet, and stressed scaliness of the feet as the most marked manifestation of the disease. Brown and Burr ('36) later observed that scaliness of the feet varied with humidity, being less severe in summer and more severe in winter. Evans and Lepkovsky ('32) observed no caudal necrosis in their animals, and they question

TABLE 1

Comparison of growth (in grams) of rats on fat-free basal diet. Curative and prophylactic values

		AVERAGE PLATEAU WEIGHT	AVERAGE FINAL WEIGHT	MAXIMUM WEIGHT	MINIMUM WEIGHT	NUMBER OF RATS
Fat-free	♂	205	165	234	137	35
	♀	171	134	177	117	35
Prophylactic dose,	♂	281	...[1]	311	251	15
10 drops of crude linoleate	♀	182	...[1]	202	146	15
Prophylactic dose,	♂	260	...[1]	287	220	25
3 drops of crude linoleate[2]	♀	190	...[1]	215	160	25
Curative dose,	♂	266	...[1]	298	204	25
10 drops of crude linoleate	♀	173	...[1]	205	129	25
Stock animals	♂	360	...[1]	425	320	Colony
	♀	220	...[1]	280	200	Colony
Burr's figures ('29)	♂	170		270[3]		
	♀	150				

[1] Maintained weight at plateau level.
[2] In Burr's experiment, lard was added to the diet.
[3] Values from a former unpublished series.

scaliness of the feet as a specific symptom because it occurred in animals receiving a diet which was not fat free.

In this series of seventy animals on a fat-free diet, diet A, approximately 20% showed caudal necrosis within 6 months. The remainder showed no macroscopic evidence of caudal involvement. Hematuria was observed in 50% of the animals, indicating gross renal damage. The two apparently infallible symptoms of the deficiency were subnormal weight and scaliness of the feet. Conditions of humidity were not studied. Alopecia was observed but in only a small percentage of cases.

The disagreement among previous observers doubtless can be explained on a basis of biological variations. This factor is indicated by differences in growth behavior as shown in this paper.

Growth behavior on fat-free diets

Table 1 is self-explanatory. It is seen that the maximum average weight of rats fed methyl linoleate prophylactically does not exceed to any significant degree the values for rats fed methyl linoleate curatively; thus indicating, if growth is used as a criterion, the fact that the fat-free regime does not permanently damage the rat under the experimental conditions observed. Several rats when fed linoleate after a 1- or

TABLE 2

Response to linoleate therapy—10 drops per day level of methyl linoleate

	RESPONSE IN 6 WEEKS		RESPONSE IN 14 WEEKS	
	♂	♀	♂	♀
	gm.	*gm.*	*gm.*	*gm.*
Average	46	23	81	31
Maximum	73	44	142	49
Minimum	15	6	47	14

Purified linoleate used during first 4 weeks; crude preparation used subsequently. There were twenty-five rats in each group.

2-month period of decline in weight failed to respond. The great biological variation shown by the rats is worthy of note.

Curves of the animals on a fat-free diet, diet A, reached a plateau in 4 to 5 months. Those for rats on diets supplemented with linoleic acid reached a second plateau in 6 to 12 months. For animals fed linoleic acid prophylactically, a plateau was reached in 7 to 9 months.

The values for animals recorded in table 2 show evidences again of a great biological variation. In the case of one male, a growth response of 200% was elicited; the animal's weight increased to double the level of the plateau reached on the fat-free diet. Variations between litters are observed.

It is to be noted from table 3 that the rate of response on the various dosage levels does not differ materially. In order

TABLE 3

Response to various dosages of methyl linoleate. The weights are measured in grams

DOSAGE	1 DROP		2 DROPS		3 DROPS		5 DROPS	8 DROPS		10 DROPS		12 DROPS	
NUMBER AND SEX OF RATS	2 ♂	2 ♀	2 ♂	2 ♀	2 ♂	2 ♀	2 ♂	2 ♂	2 ♀	2 ♂	2 ♀	2 ♂	2 ♀
Average response in 4 weeks	21	12	28	13	17	30	13	26	5	25	10	13	9
Average response in 8 weeks	38	30	48	27	32	29	27	53	34	41	23	21	12
Average response in 12 weeks	51	32	67	33	41	42	53	75	35	49	30	46	21
Total gain on indicated dose	51	32	69	35	48	45	53	77	35	50	30	48	23
Average response on 10-drop level of linoleate after plateau on indicated dosage	0 in 5 weeks	0 in 4 weeks	+7 in 5 weeks	0 in 6 weeks	2 in 6 weeks	+6 in 5 weeks	0 in 6 weeks	0 in 6 weeks	0 in 6 weeks	−1 in 4 weeks	−3 in 4 weeks	+3 in 5 weeks	−7 in 5 weeks

The linoleate fed during the first 4 weeks was prepared by the method of Rollett ('09) and subsequently our crude preparation of linoleic methyl ester was used. The rats used on the various linoleate levels had reached stationary weights at the same age.

to eliminate toxicity as a factor in this experiment, the level of methyl linoleate was doubled, 20 drops per day, in the diet of two rats, and no indications of toxicity, as shown by weight loss occurred in a period of 4 weeks. Also, to check the results for these two animals, the methyl linoleate was removed from the diet of two rats. A failure of response was taken as indicative of the absence of toxicity in the methyl linoleate as prepared and in the amount administered.

The rate of response to linoleate therapy as indicated in table 3 does not vary with dosage, but seems constant for dosages from 30 to 360 mg. of methyl linoleate daily. The total responses do not differ significantly. The lack of response to a 10-drop level of methyl linoleate after a plateau had been reached on the lower dosages (30 to 240 mg.) indicates that the optimal dosage of methyl linoleate lies somewhere below 1 drop per day per rat of the preparation used.

Six rats on the basal diet including methyl linoleate at a 10-drop level, plus all known vitamins whose curves had reached a plateau, were then given an additional supplement of a 4-drop level of methyl linolenate. The methyl linoleate used from weaning was a product prepared from corn oil by the bromination procedure of Rollet ('09). It was shown to be free of methyl linolenate. Five of the rats failed to show any response in 7 weeks; the sixth showed a slight gain. It may be concluded that methyl linolenate cannot supplement methyl linoleate in the diet of a rat. This has been assumed, but not hitherto proved.

Lard was fed at a level of 1 gm. per day per rat to five rats. The response noted was comparable with regard to time, rate of response and total gain shown by rats fed methyl linoleate. It was found impossible to obtain an added response in these rats, whose curves had reached a plateau on lard, by the addition of methyl linoleate. The reverse also held: rats stationary in weight on a diet containing methyl linoleate gave no response to the addition of lard. It is concluded that the effect of lard depends on its content of the essential unsaturated fatty acids. The samples of lard used in this work

did not produce the brain or liver effect discussed in section II of this paper. It is to be emphasized that growth responses obtained with lard are in no way superior to those obtained with pure methyl linoleate.

DISCUSSION

The contradictory state of the literature (Burr and Burr, '29, '30; Evans and Lepkovsky, '32) with regard to symptomatology in a fat deficiency disease has been explained on a basis of variation in severity with rise and fall of humidity. Further, it is possible that certain strains of inbred rats will show a difference in symptoms according to their ability to resist depletion of body stores of the essential unsaturated fatty acids. The incidence of caudal necrosis and kidney damage in the series here considered is indicative of this variation. Subnormal weight and scaliness of hind feet are the true and nearly constant indications of a fatty acid deficiency (linoleic or linolenic acid); however, even the incidence of scaly feet is not 100%.

The maximum average weights of rats fed linoleate curatively and prophylactically are comparable, thus indicating the absence of permanent damage as indicated by growth when rats are raised from weaning to plateau weights on fat-free diets. Later in the course of the deficiency disease, when the animals have lost a considerable amount of body weight, it is impossible to elicit responses even with high dosages of linoleate.

The dosages reported in the papers of Burr and Burr ('29, '30) and of Evans and Lepkovsky ('32) would seem to indicate the presence of two factors in the materials with which they were working. The fact that the minimum dosage of linoleic acid for maximum effect probably lies at or below a level of 1 drop (30 mg.) should exclude the possibility of an additional factor other than linoleic or linolenic acid present in lard. Burr's results as presented in Bull's monograph ('37) agree with our findings. However, the optimal dosage level reported by Turpeinen ('37)—100 mg. for methyl linoleate—far exceeds this value. In our experiment, four rats

were used on each dosage level. It is to be emphasized that this small number of rats does not permit an accurate statement of the minimal dosage for optimal growth effect if comparison of growth curves is used as the basis. Animals show a great biological variation in growth response. The failure of larger dosages of methyl linoleate to produce growth in animals plateaued on lower dosage levels is the basis of our statement that a minimum dosage for optimal growth effect is at or below 30 mg. per rat per day. No conclusions have been drawn from comparison of growth curves.

The evidence presented by Burr, Burr and Miller ('32) to establish the mutual replaceability of methyl linoleate and linolenate is inconclusive. They compared growth response on a 3-drop level of methyl linoleate and on a mixture of 1½ drops of linoleate plus 1½ drops of linolenate, and finding the responses comparable concluded the mutual replaceability. This evidence is not conclusive, as enough linoleate was present to give the total effect in the absence of linolenate. In order to eliminate the possibility that linolenic acid might supplement linoleic acid, we fed optimal amounts of methyl linoleate and attempted, by the addition of methyl linolenate, to obtain growth response in the animals whose weights had reached a plateau. Lack of response to the linolenic acid dosage indicated that it cannot supplement linoleic acid in the diet of the rat.

SECTION II. GROWTH EFFECT OF NATURAL DIETARY SUPPLEMENTS

From table 1 it is seen that rats fed a diet complete with regard to all known vitamins, including the essential unsaturated fatty acids, do not attain full adult size. It would seem, therefore, that the diet is still lacking in some factor essential for optimal nutrition. It was inferred that this factor would be associated with the fat fraction from natural products, as the inclusion of ether-extracted yeast provided all the water-soluble factors needed by the rat.

In recent years there have been numerous reports of growth factors outside the water-soluble group. Many of these reports seem to have overlooked the work of Evans ('28, '29)

on the growth effect of wheat germ oil. Sterol-free vitamin E concentrates showed this same growth-stimulating effect. The conclusion was that the effect was due to the vitamin E content of the materials fed. As indicated previously, vitamin E was provided in our basic diet from weaning, thus definitely excluding this factor as the source of effects observed in further study.

PROCEDURE

The weights of the animals were raised to a plateau on the diets described in section I. A plateau was said to have been reached when in 5 successive weeks the weight of the animal had remained constant or declined. It was felt that the group fed a fat-free diet, diet A, for a time might be depleted of a possible missing fat-soluble factor.

Each of the vitamin supplements was checked to assure optimal feeding levels. Blumberg ('35) has reported the completeness of this diet with respect to all known factors except vitamin E. This fact we confirmed in a series of animals, using Blumberg's technic. Vitamin E as a factor affecting growth has been excluded in this study by feeding the non-saponifiable matter of wheat germ oil equivalent to 3 gm. of the oil per week. After a plateau was reached the quantity was doubled, and, as no growth response was elicited, it was concluded that vitamin E was present in optimal amounts. The vitamin E content relative to the antisterility effect was proved by incorporating the non-saponifiable matter of wheat germ oil, at a level equal to 250 mg. per week of the oil, and noting fertility by mating tests through three successive gestations.

The supplement fed when plateau weights were reached was given at a level of 1 gm. per rat per day in every case, whether oil, fat, liver, brain, brain extract, etc. These materials were added with the regular supplements.

The oils used were pure products obtained from Eimer and Amend. The wheat germ oil was prepared fresh from wheat germ as used. The brain and liver were purchased twice weekly and preserved in a refrigerator.

RESULTS

The animals fed the diet which is complete with regard to all known dietary essentials reached stationary weights materially below the figures for stock animals. For males, the stock figure for average maximum weight is 360 gm., while for the experimental animals this value is 280 gm., approximately 75% of the stock value. For females, the stock colony value is 220 gm., while for the experimental female animals 182 is the average figure. This is 80% of the stock animal value. The weights reached a plateau in from 7 to 10 months.

It is obvious that the synthetic diet used is deficient in one or several respects. To assure the fact that animals raised on this synthetic diet were not permanently stunted, a group of twenty-three rats was placed on the McCollum stock diet. The composition of this diet follows:

Wheat (soft)	20.0
Maize (yellow)	20.0
Rolled oats	20.0
Flaxseed oil meal	10.0
Casein (crude)	3.5
Whole milk powder	25.0
$CaCO_3$	0.5
NaCl	1.0

Fe citrate (50 gm. per 100 pounds)
$CuSO_4 5 H_2O$ (20 gm. per 100 pounds)
Fresh greens—cabbage, kale, spinach, etc., 3 days a week

The response observed indicated the presence in the stock ration of some factor not present in the synthetic diet. From the composition of the stock ration, it seemed worth while to try maize oil, as maize constituted a large portion of this diet. Responses were observed as indicated in table 4. Maize oil was weakly positive. Linseed, soya bean, perilla, poppy seed and wheat germ oils were shown to be entirely negative. This rules out the factor of caloric intake as the possible explanation of the growth responses shown on various positive supplements. Liver proved considerably more potent than maize oil. Brain, however, proved the most potent source investigated, and the only source in which 100% response was observed.

TABLE 4

Response of rats on diet B with various supplements. The weights are measured in grams

SUPPLEMENT	NUMBER OF RATS		AGE LIMITS WHEN GIVEN + SUPPLEMENT IN WEEKS	PLATEAUED WEIGHTS						AVERAGE GAIN IN 4 WEEKS		AVERAGE GAIN IN 8 WEEKS		AVERAGE TOTAL GAIN		MINIMUM TOTAL GAIN		MAXIMUM TOTAL GAIN		AVERAGE FINAL WEIGHT		MAXIMUM FINAL WEIGHT		MINIMUM FINAL WEIGHT	
				Average	Maximum	Minimum	Average	Maximum	Minimum																
	♂	♀		♂	♂	♂	♀	♀	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Corn oil	9	7	31 to 45	260	306	235	171	196	147	4	2.5	8	4	16	10	—20	—9	40	26	276	181	336	216	257	155
Liver	3	4	34 to 36	275	291	251	197	201	140	17	5.3	30	9	35	14	21	5	41	24	310	211	318	218	299	164
Stock diet	10	13	28 to 38	269	284	265	167	180	151	29	17	45	26	45	26	22	18	79	39	314	193	353	209	300	173
Brain	13	11	35 to 48	273	299	242	196	205	180	23	16	43	23	45	25	32	18	70	33	319	219	343	230	288	207
Ether extract of brain	3	3	29 to 36	276	286	266	189	195	182	20	15	35	21	35	21	30	16	40	26	311	209	334	216	295	199
			AGE WHEN MADE CONTROLS																						
Controls	7	6	31 to 45	278	311	243	177	196	150	1	0	—7	2	—3	0	—17	—5	3	3	275	177	314	200	236	150

The ether solubility of the factor was demonstrated by extraction of brain in Soxhlet apparatus. The residue showed no activity, while the extract showed full potency. The factor was not water-soluble, as shown by a series of six rats whose diets were supplemented by a water extract of brain. A commercial lecithin showed no potency even when fed at a level of 0.5 gm. daily.

DISCUSSION

The report of Evans ('28, '29) in which he discusses the growth effect of wheat germ oil on vitamin E-free, sterile males seems to have been little considered in nutritional literature up to the last few years. Vitamin E was generally regarded as an antisterility vitamin and not as a growth factor. Several reports (Blumberg, '35; Martin, '37) have appeared which confirm the work of Evans by suggesting growth factors in wheat germ oil and various extracts of wheat germ oil. Evans, Emerson and Emerson ('38) using alpha tocopherol conclusively proved this point.

Numerous workers (Guha, '31; Mapson, '31; Smith and Seegers, '34; Kline, Elvehjem, Keenan and Hart, '34) report the presence in liver of a factor affecting growth. Of these, few have even considered the growth factor, vitamin E.

Work reported to date on this growth factor in liver can be criticized from three standpoints. First, the basal diets used have not been highly purified. Second, vitamin E has never been definitely excluded as a factor in the growth responses observed with liver supplements. Third, prophylactic tests have been used exclusively, fair growth being compared with good growth. The work here reported has eliminated these three possibilities of criticism. The basal diet used is highly purified. Vitamin E is provided by incorporating in the basal diet the non-saponifiable matter of wheat germ oil equivalent to 3 gm. per week per rat of the oil and is therefore excluded as the factor inducing growth response reported in these experiments. The potency of the oil for vitamin E activity was tested and shown to be high. That vitamin E

had been included in optimal amounts in the basal diet was demonstrated when we doubled the dosage and observed no weight response in animals which had reached a stationary weight with the amount of the non-saponifiable matter of wheat germ oil mentioned above. The curative method of testing was used. This eliminates the necessity of comparing growth, as we have found that biological variations in animals render inaccurate the use of prophylactic methods.

Turpeinen ('37) reports the superiority of arachidonic to linoleic acid as a curative substance in 'fat deficiency' disease. He suggests that the need of the animal organism is primarily for arachidonic acid. The bearing of this work on the results here reported is problematical. Both liver and brain contain arachidonic acid, and both exhibited the growth promoting effect observed.

The responses observed are not great but are definite. The possibility of obtaining large growth responses in rats approaching a normal for stock rats is very slight in any event. The synthetic basal diet is nearly complete. It is quite possible that the inclusion of the factor herein described in a basal diet would permit growth in rats equal to that obtained on a diet of natural foods.

No claim is made for the existence of a new vitamin. However, it is suggested that one explanation of the results reported in this paper is the existence of another vitamin.

SUMMARY

Data are presented comparing the average maximum growth of rats on fat-free diets with that of rats fed methyl linoleate curatively and prophylactically. The minimum level of methyl linoleate for optimal growth effects has been tentatively placed at or below 1 drop (30 mg.) per day per rat. The inability of methyl linolenate to supplement methyl linoleate is proved.

The observation has been made that rats fed a highly purified synthetic diet, complete in all known dietary essentials, plateau at subnormal weights. These rats show positive growth response to supplements of brain and liver.

LITERATURE CITED

BROWN, W. R., AND G. O. BURR 1936 Some recent studies in fat deficiency. J. Biol. Chem., vol. 114, p. xvi.

BLUMBERG, H. 1935 A growth deficiency disease, curable by wheat germ oil. J. Biol. Chem., vol. 108, p. 227.

BULL, HENRY B. 1937 Biochemistry of the Lipids, p. 26.

BURR, G. O., AND M. M. BURR 1929 A new deficiency disease produced by the rigid exclusion of fat from the diet. J. Biol. Chem., vol. 82, p. 345.

———— 1930 On the nature and role of the fatty acids essential in nutrition. J. Biol. Chem., vol. 86, p. 587.

BURR, G. O., M. M. BURR AND E. S. MILLER 1932 On the fatty acid essential in nutrition. III. J. Biol. Chem., vol. 97, p. 1.

EVANS, H. M. 1928–1929 The relation of vitamin E to growth and vigor. J. Nutrition, vol. 1, p. 23.

EVANS, H. M., AND G. O. BURR 1927 A new dietary deficiency with highly purified diets. Proc. Soc. Exp. Biol. and Med., vol. 24, p. 740.

———— 1927 a The antisterility vitamin fat-soluble E. U. of California, Press, Berkeley.

EVANS, H. M., AND S. LEPKOVSKY 1932 Vital need of the body for certain unsaturated fatty acids. 1. Experiments with fat-free diets in which sucrose furnishes the sole source of energy. J. Biol. Chem., vol. 96, p. 143.

EVANS, H. M., O. H. EMERSON AND G. A. EMERSON 1938 Alpha tocopherol and growth. Proc. Soc. Exp. Biol. and Med., vol. 38, p. 197.

GUHA, B. C. 1931 Observations on certain factors necessary for the normal nutrition of the rat. Biochem. J., vol. 25, p. 960.

KLINE, O. L., C. A. ELVEHJEM, J. A. KEENAN AND E. B. HART 1934 Studies on the growth factor in liver. J. Biol. Chem., vol. 107, p. 107.

MAPSON, LESLIE W. 1932 Evidence of a dietary principle stimulating general growth and lactation. Biochem. J., vol. 26, p. 970.

MARTIN, GUSTAV J. 1937 Vitamin E. J. Nutrition, vol. 13, p. 679.

ROLLET, A. 1909 Zur Kenntnis der Linolsäure. Z. Physiol. Chem., Bd. 62, S. 410.

———— 1909 a Zur Kenntnis der Linolensäure. Z. Physiol. Chem., Bd. 62, S. 422.

SMITH, H. GREGG, AND WALTER H. SEEGERS 1934 The nutritive value of animal tissues in growth, reproduction and lactation. J. Nutrition, vol. 7, p. 195.

TURPEINEN, OSMO 1937 Effectiveness of arachidonic acid in curing 'fat deficiency' disease. Proc. Soc. Exp. Biol. and Med., vol. 37, p. 37.

———— 1938 Further studies on the unsaturated fatty acids essential in nutrition. J. Nutrition, vol. 15, p. 351.

THE CONCENTRATION OF VITAMIN B_1 IN THE TISSUES OF THE RAT

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The actual function of vitamin B_1 in the animal organism is as yet imperfectly understood. In an earlier paper on vitamin B_1 metabolism (Light, Schultz, Atkin and Cracas, '38) we reported experiments on the excretion of the vitamin. We have continued our studies to determine the effect of the feeding level of vitamin B_1 on its concentration in the tissues of the rat.

Leong ('37) and Harris and Leong ('36) have reported on similar studies, using the bradycardia method of assay. Their work appeared while our studies were in progress, but it was thought advisable for us to continue in view of the difference in methods. The B_1 assay method in this and previous work is the fermentation method of Schultz, Atkin and Frey ('37 a, b, and 38). The studies on excretion of the vitamin indicated that tissue concentration of the vitamin reached a maximum at some point at or above a 65 micrograms intake per day. The results of total rat analysis tend to confirm that conclusion. To determine whether any particular organ played a major function in vitamin retention, analyses of the individual tissues of rats on both high and low vitamin levels were made.

EXPERIMENTAL

In all cases the animals were placed on a B-complex free diet composed of:

	%
Rice starch	62
Vitamin-free casein	18
Hydrogenated cottonseed oil[1]	14
Cod liver oil	2
Salt mixture	4

When indicated the vitamins of the G complex were supplied by a separate supplement of dry yeast. The vitamin B_1[2] content of this yeast (no. Y-462) was 30 micrograms per gram, and this was considered in setting the B_1 levels. Additional B_1, as the crystalline product dissolved in a 20% alcoholic solution, was given as indicated in the tables.

Light et al. ('38) showed that, when animals are continued on a uniform vitamin B_1 intake, they eventually reach a condition called excretion equilibrium, where the vitamin output in the urine remains constant and related to the intake level. In the present work all animals were brought to excretion equilibrium before analyzing. We adopted this as a criterion and thus made certain that the tissues of the animals had been adjusted to the particular feeding level at the time of analysis. In order to minimize differences due to the B_1 unabsorbed from the intestinal tract, the animals were killed 22 hours after receiving the last vitamin B_1 supplement.

TOTAL RAT ANALYSIS

For total rat analysis the carcass, after removal of the liver, was chopped well with a meat cleaver and then minced by passage through a meat grinder. The mass, including all fluids, was extracted three times with boiling acidulated water.

[1] Crisco.

[2] In this laboratory we have found by the rat growth method that 4 micrograms B_1 is equal to 1 international unit. By eluting the B_1 from the international standard and testing the eluate by the gas method, we also obtain the figure of 4 micrograms B_1 per 10 mg. of international standard. With the rat curative method, on the other hand, we have obtained 2.7 to 3.0 micrograms per unit as the ratio between crystalline B_1 and the international standard adsorbate.

The liver was treated separately in a similar manner. Three extractions in the manner described were found adequate, since two different carcasses, on analysis of the first, second and third extracts, gave 189, 42 and 7.7 micrograms respectively on one carcass, and 46, 20 and 1.5 micrograms on the other. The extracts were combined, filtered, made to volume, and sterilized in flowing steam. Table 1 gives the results obtained by anaysis of extracts so made.

The results show a gradual increase in the B_1 content of the body with increasing levels of intake up to 65 γ. Above this level there does not seem to be any increase. This result

TABLE 1

Effect of vitamin B_1 intake level on total vitamin content of the rat

VITAMIN SUPPLEMENT (PER DAY)			BODY WEIGHT (AV. OF 3 RATS)	MICROGRAMS B_1 PER GRAM BODY WEIGHT	MICROGRAMS B_1 PER GRAM LIVER
Dry yeast no. Y-462	Vitamin B_1	Total B_1			
gm.	γ	γ	*gm.*	*Average*	*Average*
0.33	..	10	118	0.54	1.08
0.66	..	20	186	0.49	1.25
0.50	15	30	198	0.92	2.8
0.50	25	40	253	1.23	4.8
0.50	35	50	244	1.34	4.85
0.50	50	65	218	1.77	6.6
0.50	50	65	149	2.14	8.15
0.50	100	115	205	1.63	5.3
0.50	500	515	195	1.88	6.8

is consistent with the observations made in our previous work (Light et al., '38) that no excretion lag could be observed at levels of 65 γ and above. This means that a rat, in excretion equlibrium at 65 γ of B_1 per day, cannot retain any more vitamin and will fall into immediate excretion equilibrium should the intake level be raised. In other words, when excretion equilibrium is reached on a 65 γ daily intake level the body tissues are saturated with respect to the vitamin.

Comparison of the data in columns 4 and 5 of table 1 shows that there is a rough inverse relationship between body weight and B_1 concentration. This may be due to a variable proportion of body fat, which on analysis has been found to be relatively low in vitamin concentration.

B_1 CONTENT OF INDIVIDUAL ORGANS AND TISSUES

For the purpose of determining the vitamin content of specific parts of the animal, the rats were anaesthetized and decapitated. The desired organs and tissues were immediately dissected out, weighed and ground in a mortar with sand. The mass so obtained was extracted three times with acidulated water at 100°C. The combined extracts were handled in the same manner as the total rat extracts described above. Table 2 gives the results obtained. The eight animals

TABLE 2

Vitamin B_1 content of organs and tissues

Animals	2 ♂		2 ♂		3 ♂ 1 ♀	
Average weight	122 gm.		245 gm.		259 gm.	
Days on test diet	28		28		28	
B_1 supplement	0		15 γ B_1 (0.5 gm. no. Y-462)		215 γ B_1 (0.5 gm. no. Y-462 + 200 γ B_1	
	B_1 γ/GM.	B_1 TOTAL	B_1 γ/GM.	B_1 TOTAL	B_1 γ/GM.	B_1 TOTAL
Liver	0.26	0.92	1.9	14.6	10.0	87.0
Heart	0.68	0.50	2.6	2.2	7.2	5.9
Testes	1.87	2.1	4.7	11.4	6.6	18.4
Kidney	0.39	0.7	2.1	3.2	6.7	11.5
Muscle (skeletal)	0.14		0.57		1.3	
Blood			0.07		0.35	

concerned in this table were of the same age and weight at the beginning of the experimental period. They had received 215 micrograms B_1 daily for 58 days, which insured complete tissue saturation with respect to the vitamins.

As might be expected the general range of tissue concentrations corresponds with the total rat concentrations at the different levels. With the lower levels of B_1 intake, the testes (1.87 to 4.7 γ/gm.) are highest in B_1 concentration. The tissue richest in B_1 at the higher levels is the liver, although all tissues have gained in vitamin content. Additional

results on the organs and tissues of saturated rats (dose 215 γ/day) were:

	γ/gm.
Body fat	0.1–0.3
Spleen	2.0–3.0
Thymus	3.0–3.5
Brain	2.5–4.2
Thyroid	4.0–6.0
Mesenteric fat	0.2–0.3

DISCUSSION

A daily dose of 65 micrograms of vitamin B_1 is sufficient to saturate the rat body. Leong ('37) reported that saturation was observed at 30 international units per day. By his conversion factor (2.9 micrograms = 1 international unit) this is equivalent to 87 micrograms. The next lower feeding level which he reported was less than 65 micrograms per day, and this did not produce saturation. Thus there is no disagreement between the results of the two investigations on this point.

Storage of vitamin B_1 in the accepted sense does not take place in the rat body. An animal whose body has been surcharged with the vitamin through the feeding of large amounts for an extended period will survive, when deprived of the vitamin, only slightly longer than another animal which had previously existed on a minimum level. This does not mean that the body content of B_1 cannot be increased, but rather that the increase is transitory and can be maintained only by constant high level feeding. The results show that at feeding levels between 10 micrograms and 65 micrograms per day the body concentration is directly related to the intake level.

It is to be noted, however, that there exists an upper limit beyond which body concentration does not go, although the actual concentration is very low in molarity. There is also unequal distribution among the organs. If we assume that the vitamin enters into a loose combination with some substance in the cell, all of our observations on B_1 retention and excretion may be explained. Such a hypothesis receives support from the coenzyme theory of vitamin B_1 function. The

vitamin molecule, probably after being phosphorylated, may be capable of combining with a specific protein or similar body to form an enzyme. Such an enzyme would, by analogy with enzymes like the dehydrogenases, readily undergo dissociation into the apoenzyme and the coenzyme. This is the loose combination mentioned above. Furthermore, it is assumed that the normal rat body will have a limited amount of the specific protein and also that organs of the body will vary according to their function in their content of that protein. The above constitutes an explanation of the maximum vitamin concentration in the body and also of its unequal distribution.

The explanation of observed phenomena is complete if it be true that the enzyme complex dissociation is an equilibrium reaction.

$$\text{Coenzyme}_{B_1} + \text{Apoenzyme} \rightleftharpoons \text{Holoenzyme}$$

If the concentration of the coenzyme is not maintained by continued feeding, then the enzyme complex dissociates and the vitamin is speedily lost to the body through excretion in the urine, or by actual destruction. When, however, the concentration of apoenzyme is high with respect to coenzyme, then the dissociation and disappearance of the complex is retarded and may virtually cease. This is found in rats deprived of the vitamin; even at the moment of death due to vitamin deficiency there is an appreciable amount of the vitamin to be found in the carcass.

The question of optimum vitamin intake is an interesting and important one. We have, so far, two fixed points in the rat's metabolism of vitamin B_1: first, the minimum daily requirement for existence and second, the minimum daily intake necessary to produce body saturation. A third point which is not yet established for the rat, using pure B_1, is that level at which the body will not absorb further vitamin B_1 from the intestine. Such a point exists in man as shown by Schultz, Light and Frey ('38). Of the three points mentioned, the one most likely to correspond to the optimum is the second. At this level (B_1 saturation) it may be said that

the physiological system of which vitamin B_1 forms a part will be able to operate at maximum efficiency, at least in so far as this vitamin is concerned. Whether such a condition actually represents the optimum, future work must determine.

The testis is the most interesting single organ which we have studied due to the tenacity with which it retains the vitamin when the animal is depleted; the significance of this is not apparent.

SUMMARY

Feeding crystalline vitamin B_1 to white rats in daily doses of more than 65 micrograms does not produce an increase in body concentration. At maximum retention (through feeding) the B_1 concentration is approximately 2 micrograms per gram of body weight.

Analyses of individual organs show a considerable difference in the concentration of vitamin B_1 therein.

The phenomenon of B_1 retention and depletion is discussed in the light of the enzyme theory of vitamin B_1 function.

LITERATURE CITED

HARRIS, L. J., AND P. C. LEONG 1936 Vitamins in human nutrition. The excretion of vitamin B_1 in human urine and its dependence on the dietary intake. Lancet, vol. 230, p. 886.

LEONG, P. C. 1937 Vitamin B_1 in the animal organism. II. A quantitative study of the metabolism of vitamin B_1 in rats. Biochem. J., vol. 31, p. 373.

LIGHT, R. F., A. S. SCHULTZ, L. ATKIN AND L. J. CRACAS 1938 The excretion of vitamin B_1 in the urine and feces. J. Nutrition, vol. 16, p. 333.

SCHULTZ, A., L. ATKIN AND C. N. FREY 1937 a A fermentation test for vitamin B_1. J. Am. Chem. Soc., vol. 59, p. 948.

———— 1937 b A fermentation test for vitamin B. II. J. Am. Chem. Soc., vol. 59, p. 2457.

———— 1938 Influence of nicotinic acid on the fermentation method for vitamin B_1 determination. J. Am. Chem. Soc., vol. 60, p. 1514.

SCHULTZ, A., R. F. LIGHT AND C. N. FREY 1938 Vitamin B_1 metabolism in man. Excretion of B_1 in urine and feces. Proc. Soc. Exp. Biol. and Med., vol. 38, p. 404.

HAIR GROWTH IN YOUNG ALBINO RATS IN RELATION TO BODY SIZE AND QUANTITY OF FOOD

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The effect of diet on body weight, length, organs, systems and teeth in rats has been studied by Jackson ('15), Winters, Smith and Mendel ('27) and Clarke and Smith ('35). Investigations (Osborne and Mendel, '14; Stewart, '16; Jackson and Stewart, '20; Jackson, '36) have also been made on the ability of rats to resume growth after inanition. Only incidental mention has been made concerning the effect of diet on hair growth.

In previous investigations (Butcher, '34, '36, '37) it has been frequently observed that the second growth of hair appeared externally on the dorsum of some rats as early as the thirty-sixth day of life, while on other rats it did not grow until they were 42 or 43 days old. Upon studying the data of previous investigations, it seemed that the factor which was in all likelihood responsible for this variation in the time of the growth of the second coat of hair was either the size of the animal at weaning time or the kind and quantity of food consumed after weaning. Accordingly, carefully planned experiments were undertaken to learn whether the size of the animal at weaning time or the food consumed after weaning modify not only the time of the second hair growth but also the quality of the hair.

The experiments have sufficed to show that, when the animals are well fed after weaning, the size of the animal (22 to

55 gm.) at weaning time does not alter the time of appearance of the second growth of hair. Hair growth is greatly affected, however, by the amount of food consumed following weaning.

METHODS

To determine if the size of the rat at weaning time was responsible for the variation in the time of growth of the second coat of hair, rats (Wistar) of varying weights were secured on the twenty-second day by limiting the number of individuals in the various litters from five to twelve soon after birth. Following weaning, the young rats were fed only dog chow.[1] The hair was removed from their backs with sodium sulphide when they were 24 to 26 days old in order to observe the time of the second hair growth. When the second growth of hair appeared externally on the dorsum, the age, nose-anus length and body weight were carefully recorded in each instance. After the hair began to grow, its quality was studied frequently.

In studying the effect on hair growth of the amount of food consumed after weaning, only rats weighing 40 gm. or more at weaning time were used. To secure such a weight the number in the litter had to be reduced to five or six soon after birth, and usually individuals were selected so that the litter consisted entirely of males or females. After weaning on the twenty-second day, the animals were isolated and were thereafter fed the dog chow. The chow checkers were finely ground to minimize the quantity of food lost from the feeding cups. One or two animals of each litter served as controls, and they had more food accessible than they consumed. Other individuals of the litter were given various quantities of food. The total quantity fed per day was divided between a feeding in the morning and one in the evening. Careful data were

[1] Purina chow, chemical analysis: protein 23%; fat 5%; fiber 4%; ash 7%; nitrogen-free extract 54%; moisture 7%. Mineral analysis: iron 0.018%; magnesium 0.09%; silica 0.23%; potassium 0.56%; sodium 0.67%; chlorine 0.68%; phosphorus 1.17%; calcium 2.22%; sufficient iodine. Vitamin content: A, 4000 international units per pound; B, 275 Sherman units per pound; C, low; D, 500 international units per pound; E, supplied by wheat germ; G, 300 Sherman units per pound.

kept on the body weight and length at the time of the appearance of the second growth of hair in all rats, and this is recorded in table 2.

The effect of the size of the animal at weaning time on hair growth

As may be seen in table 1, the body weight at weaning time varied from 22 to 55 gm. When the rats were fed an unlimited quantity of food after weaning, the weight of the rat at weaning time had no effect on the time of appearance of the second growth of hair, which usually appeared externally in most rats at the age of 37 days. In two litters, fortunately, the animals ranged in weight from 22 to 34 gm. at weaning time. The second coat of hair grew on the dorsum of these rats about the same time. Body weight at the time of appearance of the second growth of hair, the animals usually being 37 or 38 days old, ranged from 54.5 to 129 gm. Therefore, the body did not have to reach a certain weight before the hair grew.

The time of the second hair growth closely correlates with age but with neither body length nor weight. The coefficient of correlation of body length with the time of growth for the ninety-three animals in table 1 equals —0.04; body weight with the time of growth equals —0.127; body length with body weight equals 0.854. These results on hair growth in rats agree with those on rate of feathering in chicks, since Warren ('25) has found that the vigor of chicks has no influence on the rate of feathering.

The effect of the amount of food on hair growth after weaning

Rats weighing 40 to 47 gm. at weaning time

At the beginning of these experiments, it was found that rats gained very little in body weight when fed only 4 gm. of the finely ground chow per day following weaning. Accordingly, it seemed inadvisable to feed a smaller quantity than 4 gm. lest the physical condition of the rat would be too greatly affected.

TABLE 1

The effects of the size of the animal on the time of appearance of the second growth of hair

NUMBER OF ANIMALS	TWENTY-SECOND DAY WEANING				WHEN SECOND GROWTH OF HAIR APPEARED					
	Range of weight	Average weight	Range in length nose-anus	Average length	Age range	Average age	Range in weight	Average weight	Range in length	Average length
	gm.	gm.	mm.				gm.	gm.		
10 ♀	22.5–29.5	26.5	92–101	96.6	37–39	37.7	54.5–71	66	127–137	133
8 ♂	27.5–29.5	28.6	97–103	101	37–38	37.5	65–79	73	140–145	143
16 ♀	32–38	34.2	103–106	104	36–40	37.5	65–91	80.6	135–143	140
9 ♂	32.5–38.5	35.5	104–108	106	36–39	37.3	71–95	83	140–150	146
16 ♀	41–48	43.6	105–110	106	36–41	37.5	67.5–104.5	90.2	142–150	145
17 ♂	41–48	43.8	108–112	110	36–41	38.6	75.5–129	102.3	143–152	147
6 ♀	50–55	52	115–117	115.8	36–38	37	100–112	104	151–162	156
11 ♂	50–52	51	115–121	117	37–40	38.3	84–122	107	148–163	156

TABLE 2

The effect of the quantity of feed on hair growth in young rats

EXPERIMENT	NUMBER OF ANIMALS	AVERAGE WEIGHT ON 22ND DAY	AVERAGE LENGTH NOSE-ANUS	GRAMS OF FEED DAILY UNTIL HAIR APPEARED						WHEN HAIR APPEARED			
				22–24	25–27	28–30	31–33	34–36	37–	Average amount fed	Average age	Average weight	Average length
		gm.	mm.										
1	7 ♀	41	103	4	4	4	4	4	4[1]	196	72	54.6	132
2	3 ♂	43	108	4	4	4	4	4	4[1]	204	74	57	132
3	3 ♀	53.5	117	4	4	4	4	4	4[1]	224	77	58	133
4	5 ♀	43.6	108	5	5	5	5	5	5[1]	195	62	71.6	139
5	2 ♂	42	108	5	5	5	5	5	5[1]	175	58	69.2	138
6	3 ♂	51	115	5	5	5	5	5	5[1]	85	40	77.5	148
7	3 ♀	43.5	106	4	4	5	5	6	6[1]	120	45	66.6	135
8	3 ♂	52.5	117	4	4	5	5	6	6[1]	90	40	79	148
9	3 ♂	43	107	6	6	6	6	6	6[1]	108	41	70.6	141
10	3 ♀	43	108	6	6	6	6	6	6[1]	102	40	73	142
11	3 ♂	47	111	4	5	6	7	8	9[1]	117	40	95	151
12	3 ♀	45	108	4	5	6	7	8	9	99	38	92	147
13	3 ♂	42	106	5	6	7	8	9	10	105	38	88.3	148
14	4 ♀	41.5	104	5	6	7	8	9	10	105	38	83.2	142
15	9 ♂	44.2	110	Abundance of feed						117.4	37	105	150
16	8 ♀	44	106	Abundance of feed						116.8	37	94	146

[1] The last amount was continued daily until the hair appeared.

The second growth of hair on rats, fed only 4 gm. per day, appeared externally when the rats averaged 73 days in age (experiments 1 and 2, table 2). Growth in no instance occurred before the age of 61 days, and no later in any animal than the eighty-fifth day. The hair appeared unevenly over the dorsum and grew slowly so that bare areas persisted on the backs of the rats. Histological sections of skin taken from the dorsum of underfed animals 50 days old showed that the hair bud of the second hair had not begun to grow. Since the bud becomes active about the thirty-second day in well-fed rats, underfeeding had greatly retarded growth. After activity begins in the hair bud, histological observations show that growth of the hair progresses much more slowly in the underfed rats than it does in well-nourished animals.

Five grams of feed per day, 2 in the morning and 3 in the evening, induced hair growth usually when the rats were 60 days old which is about 13 days sooner than when 4 gm. were fed daily (experiments 4 and 5, table 2). The hair, however, appeared in patches externally on the dorsum, and it was 20 days after the first appearance of the hair before the back was completely covered. In one 43-day-old rat (experiment 5) a small area of hair appeared. No more grew until the age of 56 days. The increase to 5 gm. of food per day resulted in longer and heavier animals before hair growth was induced.

Beginning with 4 gm. and then increasing the diet of rats 1 gm. every 6 days (experiment 7) resulted usually in small areas of growth, 1 cm. in diameter, at the age of 45 days. Hair then appeared slowly over the rest of the back, but for 15 days bare areas could be seen. The slight growth on the forty-fifth day indicated that the cyclic activity of the hair coat was being attempted, but that the stimulus was insufficient to induce hair growth over all of the back.

Six grams of feed per day (experiments 9 and 10) induced hair growth on the dorsum quite similar to the growth obtained in experiment 7, where the quantity was increased every 6 days. The hair appeared in patches on 40- and 41-day-old rats, and regions of the back remained nude as late

[1] The last amount was continued daily until the hair appeared.

as the fifty-ninth day of life. Body length was affected relatively less than body weight by the food insufficiency. When the rats were given an unrestricted quantity of food after the first appearance of the hair on the forty-first day, hair growth over the back progressed rather evenly.

In experiments 11, 12, 13 and 14 the food intake was practically sufficient to induce the same body length and hair growth as that on animals allowed all they could eat.

Rats weighing 50 gm. or more at weaning time

When 22-day-old rats weighed 50 gm. or more, and were fed only 4 gm. daily, the second hair appeared at the average age of 77 days (experiment 3, table 2). This was about the same time as when the weight of the rat was 40 gm. at the beginning of the experiments. Fifty-gram rats, receiving 5 gm. of feed per day, produced hair externally at the age of 40 days (experiment 6), which was much earlier than the growth on those rats which weighed 42 gm. at weaning and received 5 gm. of food daily (experiment 5). With an increase of food every 6 days in 50-gm. rats (experiment 8), the hair grew on 39- and 40-day-old rats, just 3 days later than on litter mate controls. The hair appeared rather evenly over the dorsum but its growth afterward was greatly retarded.

Further experiments with larger amounts of food were not conducted on rats weighing 50 gm. at weaning time since the hair on such animals receiving 5 gm. daily had grown almost at the regular time.

In these experiments on the effect of the amount of food on hair growth after weaning it is again shown that there is no correlation between body size and the time of hair growth since the coefficient of correlation of body length with the time of hair growth for the sixty-five animals in table 2 equals -0.76; weight with the time of hair growth equals -0.644. The coefficient of correlation for length with weight, however, is 0.784.

DISCUSSION

By cell proliferation the hair bud is able to produce a hair. For this growth the hair bud must have building materials, stimulation and receptivity. Which of these factors is the most important and which is affected most by the underfeeding presents an interesting problem.

Although the building materials may be present, the hair will not grow without some other factor. This is shown by the cyclic activity of the hair coat (Butcher, '34), and by the present experiments on the effect of size at weaning time on hair growth. The hair grew no sooner on the heavier animals.

In the experiments on underfeeding the hair failed to grow in animals which often reached a greater size than rats in which hair growth had been induced by ample food. Other structures, such as the skeleton, grew in these underfed animals, and building substances were therefore present for growth. Waters ('08) likewise found that the skeleton was less influenced than other parts by inadequate diets, and that it persisted in growth when the growth of the animal as a whole had ceased. Thus it seems quite probable that the retardation of hair growth in these underfed animals is not due to insufficient building substances. Either the stimulus or the receptivity of the hair bud must have been impaired by the underfeeding.

By transplantation experiments (Butcher, '36) it was learned that the hair bud was receptive most of the time. In the present underfeeding experiments hair grew at different times and in patches which would indicate that some hair buds were receptive, and there is every reason to believe that all of them in the dorsum were equally receptive.

Eliminating receptivity and lack of building material as the primary causes for the retardation of hair growth upon underfeeding, impairment of the stimulus would seem to be left as the probable cause. If endocrine glands are responsible for initiating the cyclic activity of the hair coat, they may be altered by the insufficiency of nutrition, and not supply the stimulus for hair growth. Possibly changes in the adrenal

may account for the retardation. Vincent and Hollenberg ('20) found that in underfed rats, the adrenal hypertrophies greatly. In my experiments ('37) it was shown that adrenalectomy induced an early second growth of hair in the rat, and that this earlier growth was due to the removal of the adrenal's inhibitory influence on the thyroid. It is possible, therefore, that the great retardation of hair growth in underfed animals is due to a disturbed adrenal-thyroid relationship. This problem is being investigated.

In some instances the quantity of food was sufficient to start hair growth at the regular time. The hair then grew slowly, sparsely, and the quality was poor. Therefore, not only is the time of appearance of the hair affected by inadequate food but also the general growth of the hair.

The question arises whether the retarded effect produced by 4 gm. per day has or has not any permanent effect on hair growth. If the daily amount of 4 gm. is continued, sparseness and difference in quality can be noted. If the animal is given all the food that it can consume after the hair starts to grow there seems to be little effect on the buds or the character of the hair.

CONCLUSIONS

1. The size at weaning time (22 to 55 gm.) does not alter the time of appearance of the second growth of hair in the albino rat, providing they are well fed after weaning.

2. After weaning underfeeding greatly retards the rate of hair growth in the rat. Following the initial appearance, the hair grows unevenly and slowly over the remainder of the back.

3. Impairment of the stimulus which induces hair growth seems to be the most probable cause for the retardation of hair growth upon underfeeding.

LITERATURE CITED

BUTCHER, E. O. 1934 The hair cycles in the albino rat. Anat. Rec., vol. 61, p. 5.

———— 1936 Hair growth on skin transplants in the immature albino rat. Anat. Rec., vol. 64, p. 161.

———— 1937 Hair growth in adrenalectomized, and adrenalectomized thyroxin-treated rats. Am. J. Physiol., vol. 120, p. 427.

CLARKE, M. F., AND A. H. SMITH 1935 Effect of a diet poor in salts upon the growth and composition of the incisors of the rat. Am. J. Physiol., vol. 112, p. 286.

JACKSON, C. M. 1915 Effects of acute and chronic inanition upon the relative weights of the various organs and systems of adult albino rats. Am. J. Anat., vol. 18, p. 75.

———— 1936 Recovery in rats upon refeeding after prolonged suppression of growth by dietary deficiency of protein. Am. J. Anat., vol. 58, p. 179.

JACKSON, C. M., AND C. A. STEWART 1920 The effects of inanition in the young upon ultimate size of the body and of various organs in the albino rat. J. Exp. Zool., vol. 30, p. 97.

OSBORNE, T. B., AND L. B. MENDEL 1914 The suppression of growth and the capacity to grow. J. Biol. Chem., vol. 18, p. 95.

STEWART, C. A. 1916 Growth of the body and of the various organs of young albino rats after inanition for various periods. Biol. Bull., vol. 31, p. 16.

VINCENT, S., AND M. S. HOLLENBERG 1920 Changes in the adrenal bodies and the thyroid resulting from inanition. J. Physiol., vol. 54, p. lxix.

WARREN, D. C. 1925 Inheritance of rate of feathering in poultry. J. Heredity, vol. 16, p. 13.

WATERS, H. J. 1908 The capacity of animals to grow under adverse conditions. Proc. Soc. Promotion Agri. Sci., twenty-ninth annual meeting, p. 71.

WINTERS, J. C., A. H. SMITH AND L. B. MENDEL 1927 The effects of dietary deficiencies on the growth of certain body systems and organs. Am. J. Physiol., vol. 80, p. 576.

A LONG-TIME STUDY OF NITROGEN, CALCIUM AND PHOSPHORUS METABOLISM ON A LOW-PROTEIN DIET [1]

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In human balance experiments, daily fluctuations in excreta, even on a constant diet, have been frequently observed. Martin and Robison ('22), studying the biological value of proteins, reported nitrogen fluctuations amounting to 25% of the mean output on a minimum nitrogen diet, in spite of constant food and fluid intakes and regular habits.

To insure reliability of results in nitrogen balance experiments, Boas-Fixsen ('35) emphasized among other things the necessity of a sufficiently long pre-period on the diet to be studied and a long experimental period.

In contrast to this idea, Pyle and Huff ('36) found in calcium and phosphorus studies of pregnant women, that 1-day balances following 2-day pre-periods did not differ significantly from results of Macy et al. ('30) and Coons and Schiefelbusch ('34) with longer periods. Porter-Levin's ('33) findings with pre-school children indicated that 15 to 21 consecutive days of balance were required to cover the range of variation in retention of nitrogen, calcium and phosphorus. Hawks et al. ('37) also working with pre-school children, used 10- to 12-day preliminary periods and collection periods of 21 to 24 days, believing this time was sufficient to show "trends in the metabolism and the 3-day period variations."

[1] Contribution no. 81.

A tendency to favor long-time balance experiments is evident in these and other studies. Hummel et al. ('36) in their studies on pregnant women say, "It has become increasingly apparent that it is expedient to extend the short balance to a longer period of time . . . ."

Believing that a cyclic retention of nitrogen, calcium and phosphorus might be indicated in a long-time study of women, a 45-day balance experiment was planned in which three young women, 23 and 24 years of age, served as subjects.

METHODS

The three subjects, M, B and A, weighing 49.4, 50.1 and 55.9 kg., respectively, were normal as determined by physical examinations. They were fairly active and were all engaged in graduate study involving similar activities and interests.

A moderately low-protein diet (table 1) was planned to provide 45 Calories per kilogram and to supply 75% of the protein requirement of each subject. Meat was to furnish 85% of this protein. During a 3-day pre-period on the diet the desired distilled water intake was set by each subject at 1100 cc. and the NaCl, at 5.0 gm. daily. The subjects ate the diet for fifteen consecutive 3-day periods.

With the exception of butter, sufficient quantities of the foods to last throughout the diet were purchased at one time and held at suitable temperatures. Butter was churned in two lots and stored below 0°C. The meat, U. S. good beef round,[2] was freed from visible fat, ground three times with a coarse knife, thoroughly mixed and analyzed for nitrogen. On the basis of this analysis the amount of meat needed for the entire study by each subject to supply the desired daily nitrogen was determined. These amounts were then divided into two daily portions of equal weight for each subject and stored below 0°C. until used. The fruit punch, consisting of 150 gm. orange juice and 350 gm. grape juice for a subject, was prepared daily. Sufficient orange juice for the day's needs was expressed, strained and aliquot portions weighed.

[2] Purchased from the department of animal husbandry of the Kansas State College.

The necessary amount of grape juice was opened, mixed and weighed portions combined with the orange juice. Sucrose and lactose were added in varying amounts for the different subjects in order to adjust the Calories to individual needs. Cabbage sufficient for 3 days was shredded on the first day of a period, stored in an ice box in tightly closed jars, and portions weighed daily as needed. Subjects M and B used a salad dressing of corn oil and dilute citric acid with the cabbage. Subject A disliked oil so increased butterfat and sugar for the necessary Calories. A 'cracker' containing agar, and practically nitrogen-free was made according to the recipe of Long and Pittman ('35).

Breakfast consisted of fruit punch, cracker, butterfat and water. Mineral supplements nearly sufficient to meet the Sherman standards ('37) were taken at this time (table 1). The noon and evening meals consisted of the meat, cabbage salad, fruit punch, cracker and butterfat. The frozen meat for a single meal was allowed to come to room temperature, then baked at 350°F. in individual covered casseroles with a little distilled water and half the subject's daily supply of salt. The meat was served in the casserole which the subject afterward rinsed carefully with a portion of her distilled water to minimize loss.

The daily aliquots of punch were mixed and canned at the end of each period for subsequent analysis. Representative portions of cabbage for a period were mixed, dried in an oven at 80°C., then powdered and stored until analyzed. A sample of meat was preserved in the same way as the cabbage. Crackers, salad oil and filtered butterfat were not analyzed as they were thought too low in nitrogen and minerals to affect results.

Excreta were analyzed by periods. The 24-hour urine was collected under toluol and chilled until the day's collection was complete. It was then mixed and an aliquot for each day's total volume, acidified with HCl, retained for the period-composite. Total urinary acid and pH were determined at the end of each 24-hour day. Carmine was used to mark the

TABLE 1

Daily food intakes on a low-protein diet [1]

SUBJECT	COMPOSITION OF DIET	BEEF ROUND	BEVERAGE	CRACKERS	BUTTER FAT	CABBAGE [2] SALAD	$FeC_6H_5O_7 \cdot 3H_2O$	KH_2PO_4	$CaH_4(PO_4)_2 \cdot H_2O$	NaCl	TOTAL
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
M	Weight	92.4	600.0 [3]	198.2	54.0	102.1	0.0463	0.548	2.082	5.000	
(49.4 kg.)	Nitrogen	3.24	0.42			0.19					3.85
	Calcium	0.005	0.059			0.038			0.331		0.433
	Phosphorus	0.177	0.067			0.023		0.125	0.512		0.904
	Fiber			2.7		0.8					3.5
	Ml. excess N. acid	12.85						8.05	33.00		53.90
	Ml. excess N. base		22.07			3.45	0.46				25.98
B	Weight	96.6	600.0 [3]	198.2	53.5	113.1	0.0458	0.548	2.214	5.000	
(50.1 kg)	Nitrogen	3.38	0.42			0.19					3.99
	Calcium	0.006	0.059			0.038			0.352		0.455
	Phosphorus	0.185	0.067			0.023		0.125	0.545		0.945
	Fiber			2.7		0.8					3.5
	Ml. excess N. acid	13.43						8.05	35.10		56.58
	Ml. excess N. base		22.07			3.45	0.46				25.98
A	Weight	102.5	620.0 [3]	297.0	59.0	79.4	0.0449	0.526	2.403	5.000	
(55.9 kg.)	Nitrogen	3.57	0.42			0.19					4.18
	Calcium	0.006	0.059			0.038			0.382		0.485
	Phosphorus	0.196	0.066			0.023		0.120	0.591		0.996
	Fiber			4.0		0.8					4.8
	Ml. excess N. acid	14.26						7.72	48.10		70.08
	Ml. excess N. base		22.07			3.45	0.46				25.98

[1] Based on analysis of foods by periods.

[2] All subjects had 79.4 gm. shredded cabbage; subject M had 22.2 gm. corn oil and 0.7 gm. citric acid; subject B had 33.0 gm. oil and 0.7 gm. citric acid.

[3] 150 gm. strained orange juice, 350 gm. commercial grape juice, 50 gm. lactose, 50 gm. sucrose.

[4] Same as for subject M except 65 gm. lactose and 55 gm. sucrose.

fecal periods. The combined feces for a period were mixed with acidified alcohol, dried below 80°C., then ground.

Analyses of food and excreta were made in triplicate. Nitrogen was determined by the Gunning-Arnold modification of the Kjeldahl procedure: calcium, volumetrically by a method used in the Kansas Agricultural Experiment Station; and phosphorus, gravimetrically by the Neumann method ('02–'03) as modified by Lundell and Hoffman ('23) and McCandless and Burton ('24). Total acid was determined by the Folin method (Hawk and Bergeim, '31) and pH by means of a quinhydrone electrode according to Billmann and Tovborg-Jensen ('27).

RESULTS

The subjects remained in good health and spirits throughout the experiment, doubtless aided to a considerable extent by their interest in the study. The caloric intake was sufficient to maintain weight in all cases. The cracker was the least palatable item in the diet but no serious complaint was voiced. Analysis of the food items showed but little variation in the nitrogen, calcium and phosphorus intakes for the different periods so the diet was regarded as practically constant.

Nitrogen findings. As the meat had been analyzed for nitrogen before the diets were calculated and the other foods contained approximately the amounts of nitrogen estimated, the food actually supplied 75% of the protein requirement for each subject (0.42 gm. per kilogram) with 84.8% of the total nitrogen furnished by meat.

The average daily nitrogen intakes (table 2) were 3.85, 3.99 and 4.18 gm. for subjects M, B and A, respectively, or expressed in milligrams per kilogram, 77.9, 79.6 and 74.8 with standard deviations on the latter basis of only 0.71, 0.70 and 0.72 mg. The maximum variation in intake of nitrogen during the fifteen periods was 2.4 mg. per kilogram for subject M and the minimum was 2.2 mg. for subject A.

The mean dry weight of the feces was 305.8 mg. per kilogram per day. Of this amount, 16.7 mg. were nitrogen which was 21.6% of the total mean nitrogen intake (77.4 mg. per kilogram

per day), making the average apparent coefficient of digestibility for the nitrogen 78.4% for the three subjects during the study. The range in digestibility was from a mean of 77.9 to 78.8% for the various subjects although variations from period to period ranged from a low of 68.4% for subject B in period 2 to a high of 88.1% in period 10 for subject A. The average nitrogen absorption for subjects M, B and A was 61.1, 62.1 and 60.0 mg. per kilogram.

TABLE 2

Average daily nitrogen, calcium and phosphorus balances on a low-protein diet

SUBJECT	AVERAGE DAILY INTAKE		AVERAGE DAILY OUTPUT			BALANCE	
	Meat	Total	Urine	Feces	Total	Total	PER KILOGRAM
			Nitrogen				
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*
M	3.24	3.85	2.48	0.83	3.31	+0.54	+10.9
B	3.38	3.99	2.92	0.88	3.80	+0.19	+ 3.8
A	3.57	4.18	2.83	0.87	3.70	+0.48	+ 8.6
Mean	3.40	4.01	2.74	0.86	3.60	+0.40	+ 7.8
			Calcium				
M	0.005	0.433	0.110	0.377	0.487	−0.054	− 1.1
B	0.006	0.455	0.154	0.400	0.554	−0.099	− 2.0
A	0.006	0.485	0.105	0.470	0.575	−0.090	− 1.6
Mean	0.006	0.458	0.123	0.416	0.539	−0.081	− 1.6
			Phosphorus				
M	0.177	0.904	0.571	0.328	0.899	+0.005	+ 0.1
B	0.185	0.945	0.644	0.360	1.004	−0.059	− 1.2
A	0.196	0.996	0.701	0.322	1.023	−0.027	− 0.5
Mean	0.186	0.948	0.639	0.337	0.975	−0.027	− 0.5

Individual differences were noted in fecal nitrogen. It was most constant for subject B varying only from 55.1 to 58.7 mg. per gram dry feces for any given period, a difference of 3.6 mg. per gram. Subject M showed most variation ranging from 38.6 to 57.7 mg. per gram. The average was 54.9 mg. per gram for the three subjects with A and B exceeding the average and M falling below it.

Urinary nitrogen averaged 68.3% of the nitrogen intake varying from a mean of 73.2% for subject B to 64.4% for subject M. The mean nitrogen output in urine and feces totaled 70 mg. per kilogram, an average of 90% of the intakes of the three subjects.

The nitrogen balances indicating body retention (table 2) show a mean for all subjects of +0.40 gm. per day or 7.8 mg. per kilogram. Subjects M and A were in positive balance

TABLE 3

Average daily nitrogen, calcium and phosphorus retentions per kilogram on a low-protein diet

PERIOD	SUBJECT M			SUBJECT B			SUBJECT A		
	N	Ca	P	N	Ca	P	N	Ca	P
	mg	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	7.3	0.7	1.6	−13.1	− 3.3	−2.6	±0.0	− 4.3	−3.3
2	16.4	− 1.6	±0.0	− 7.0	− 3.5	−4.5	5.3	− 1.4	−1.1
3	19.3	− 0.3	−0.3	3.8	0.3	0.5	8.1	− 2.2	−1.1
4	14.2	− 1.9	−0.1	2.2	− 1.8	−2.4	9.2	0.9	1.0
5	16.0	1.1	2.2	13.7	− 1.7	−1.4	4.4	− 2.5	−0.8
6	6.6	1.6	2.0	− 2.0	− 1.0	−2.4	8.5	− 1.3	−0.2
7	4.1	− 3.1	−1.5	− 1.8	− 4.2	−4.6	10.5	− 3.5	−1.2
8	5.0	− 1.4	−0.1	3.3	− 2.1	−0.6	12.7	− 2.7	−0.1
9	12.0	− 4.5	−3.0	8.8	− 3.4	−2.6	0.3	− 3.2	−0.8
10	12.9	− 1.2	−0.8	17.1	− 1.3	2.1	13.2	1.5	2.0
11	4.6	− 2.1	−1.9	± 0.0	− 3.4	−2.2	10.9	0.5	−0.5
12	16.6	0.9	1.3	4.9	− 1.6	0.5	15.7	− 1.2	0.6
13	8.0	− 3.2	0.8	10.0	0.2	1.9	15.9	− 0.6	0.2
14	12.5	− 1.0	−0.5	5.6	− 2.4	−0.3	10.7	− 3.1	0.1
15	7.7	− 0.4	1.6	12.2	− 0.4	0.7	4.3	− 0.3	−2.5
Mean	10.9	− 1.1	0.1	3.8	− 2.0	−1.2	8.6	− 1.6	−0.5
				Per cent of intake					
Mean	14.0	−12.5	0.5	4.8	−22.0	−6.4	11.5	−18.4	−2.8

throughout the fifteen periods (table 3). Subject B was in decidedly negative balance in periods 1 and 2 and slightly so in periods 6 and 7. Her average for the fifteen periods was close to equilibrium with +0.19 gm. per day or a retention of 3.8 mg. per kilogram which was 4.8% of her nitrogen intake. In contrast, subject M retained 0.54 gm. per day or 10.9 mg. per kilogram which was 14.0% of her intake. Subject A fell between, with a mean retention of +0.48 gm. nitrogen daily or 8.6 mg. per kilogram which amounted to 11.5% of her intake.

The mean nitrogen retention on this low-protein diet of 7.8 mg. per kilogram for these young adult women was surprisingly high, approximating one-fourth of that reported by Hawks et al. ('38) for pre-school children on a medium-protein diet. This high retention does not appear to be explained as the result of a nitrogen-poor condition of the subjects previous to the study, for storage tended to increase as the study proceeded. It is also surprising that there was no tendency in these presumably mature subjects to approach nitrogen equilibrium in 45 days on a constant diet.

The average daily calcium intakes were 0.433, 0.455 and 0.485 gm. (table 2) for subjects M, B and A, respectively, which amounted to 8.8, 9.1 and 8.7 mg. per kilogram with standard deviations of 0.20, 0.19 and 0.19 mg. The maximum variation in calcium intake was 0.61 mg. per kilogram for subjects M and A and the minimum was 0.58 mg. for subject B. On the whole the calcium intakes were regarded as relatively constant.

The mean calcium output was 0.539 gm. per day or 10.4 mg. per kilogram. The average daily fecal output was 8.0 mg. per kilogram which was 77.0% of the total output. Subject A, with a more bulky diet, excreted 8.4 mg. calcium per kilogram by way of the feces, somewhat more than the other subjects.

Urinary calcium for the three subjects averaged 2.4 mg. per kilogram. Subject B excreted 3.1 mg. calcium through the urine while subject A, with more bulk, combined a relatively low urinary calcium (1.9 mg. per kilogram) with high fecal calcium.

The average calcium retention was negative for all subjects, the mean for the group being —1.6 mg. per kilogram or —0.081 gm. per day. Subject B was most negative averaging —2.0 mg. per kilogram per day (table 3). Subjects B and A were in positive calcium balance for only two and subject M for three of the fifteen periods of the study.

The average daily phosphorus intakes for the three subjects M, B and A were 0.904, 0.945 and 0.996 gm. per day (table 2) or 18.3, 18.9 and 17.8 mg. per kilogram, respectively, and

averaged 18.3 mg. per kilogram. These intakes were remarkably constant showing standard deviations of only 0.24, 0.24 and 0.19 mg. The average fecal phosphorus constituted 35.6% of the intake or 6.5 mg. per kilogram and ranged from a low of 5.8 mg. per kilogram for subject A to a high of 7.2 for subject B.

For subjects M, B and A the urinary phosphorus was 11.6 12.8 and 12.5 mg. per kilogram and averaged 12.3 mg. or 67.4% of the intake. These results are in line with other findings. Porter-Levin ('33) reported a slightly larger portion of total phosphorus excretion in the urine and somewhat smaller amount in the feces in the 2-, 4- and 5-year-old children she observed. Davis ('35), studying older children, reported averages of 42.7% of the total phosphorus excretion in the feces and 57.3% in the urine on an acid diet.

Shohl and Sato ('23) in a study of two infants reported excretion of phosphorus largely by way of the urine which in normal periods averaged 68.5 to 65.0%. They also found that addition of acid shifted phosphorus excretion from stool to urine. Meyers and Fine ('19) in a study of adults reported two-thirds of the phosphorus in the urine and one-third in the feces. The total daily excretion of phosphorus for subjects M, B and A amounted to 18.2, 20.0 and 18.3 mg. per kilogram, averaging 18.8 mg. and resulting in a mean balance for all subjects throughout the study of —0.5 mg. per kilogram. The average daily retention for subjects M, B and A was +0.1, —1.2 and —0.5 mg. per kilogram and the number of negative balance periods were 9, 10 and 10 (table 3), respectively, indicating that the phosphorus was not well utilized at this level. The range of phosphorus retention for the three subjects was from +2.2 mg. per kilogram to —3.0 for subject M, +2.1 to —4.6 for subject B, and +2.0 to —3.3 for subject A.

The diet was acid-forming, having a daily excess of 27.9, 30.6 and 44.1 ml. for N acid for subjects M, B and A. However, the reaction of the diet probably was not sufficiently different from an ordinary one to influence calcium and phosphorus partition greatly.

There was considerable variation in total urinary acid from period to period for each subject and the average excretions differed. The total urinary excretion for subject M was 141.4 ml. of N/10 acid whereas subject B excreted 225.3 ml. and A, 235.3 ml. Some fluctuation was also noted in the urinary pH readings and a slight tendency toward increased acidity throughout the 45 days whereas no change in trend of total acid excretion could be observed. The diet in this experiment contained 350 gm. grape juice and 150 gm. orange juice. Clouse ('35) found that 500 gm. of either grape or orange juice reduced total acid excretion but that grape juice had a less marked effect. She also found a rise in urinary pH with this quantity of orange juice but observed no change when grape juice was used.

The lactose in the diet was probably not a factor in the usage of the calcium and phosphorus. The subjects were not in a growing stage and the quantities used were relatively small.

Six menstrual periods occurred during the study, two for each subject, but the menstrua were not collected. During these periods nitrogen and calcium appeared to be retained in amounts above average in four instances and below in one. Phosphorus was retained in amounts above average in only half of the periods, the other half being below the mean. The unfavorable retention periods were distributed among the three subjects, no one being particularly adversely affected. In no case were the extreme-low points of the study reached at this time.

Basal metabolism tests were made weekly on each subject. The averages of the six tests, expressed in terms of the Aub-DuBois standard as modified by Boothby and Sandiford, were +3.34, —6.72 and —12.42 for subjects M, B and A. No abnormalities were apparent although subject M's may be regarded as rather high. The basal rates showed no consistent relation to menstruation, which agrees with Blunt and Dye ('21). They were high during both periods for subject M at this time and either average or below for the others.

The average per capita intake of fiber, chiefly in the form of agar, was 3.9 gm. daily (table 1). Subjects M and B each consumed 3.5 gm. and A, 4.8 gm., 37% more than the other subjects. In spite of higher fiber intake, subject A had a smaller volume of dry feces with an intermediate value for nitrogen per gram and the lowest fecal nitrogen expressed as milligram per kilogram. Fecal calcium was high and urinary calcium low for A in comparison with the other subjects, although total calcium output was similar. In contrast to the results with calcium, fecal phosphorus was low but total phosphorus output was also close to the average for the three subjects. As the fiber content of all three diets was low, it is doubtful if in any case it had an important influence on the results.

CONCLUSIONS

1. In all subjects considerable variation in retention of nitrogen, calcium and phosphorus was noted from period to period throughout the 45 days of the study. No definite cyclic tendency was apparent.

2. Measurement of the utilization of nitrogen, calcium and phosphorus in young women subjects on moderately low protein diets requires a long-time consecutive balance study, the length of time probably depending on the subject.

3. When meat furnished 85% of the nitrogen of a low-protein diet, the average nitrogen balance for fifteen consecutive 3-day periods was positive for each subject. In two cases there was a trend toward increased nitrogen retention throughout the study although both were presumably adults (23 and 24 years).

4. Levels of 92 and 97% of the commonly accepted standards of 0.68 gm. calcium and 1.32 gm. phosphorus per 70 kg. were not sufficient to prevent losses in the three young women subjects of this study in which protein was fed at 75% of the requirement. The fluctuations in retention of these two minerals tended to parallel, but phosphorus was better used than calcium.

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LITERATURE CITED

BILLMAN, E., AND S. TOVBORG-JENSEN 1927 On the determination of the reaction of soils by means of the quinhydrone electrode. Trans. 2nd Comn. Internatl. Soc. Soil Science B: 236.

BLUNT, K., AND M. DYE 1921 Basal metabolism of normal women. J. Biol. Chem., vol. 47, p. 69.

BOAS-FIXSEN, M. A. 1935 The biological value of protein in nutrition. Nutr. Abst. and Rev., vol. 5, p. 447.

CLOUSE, R. C. 1935 Effect of grape as compared with other fruit juices on urinary acidity and the excretion of organic acids. J. Nutrition, vol. 9, p. 593.

COONS, C. M., AND A. T. SCHIEFELBUSCH 1934 Acid-base balance of the minerals retained during human pregnancy. J. Biol. Chem., vol. 104, p. 757.

DAVIS, N. J. 1935 Calcium, phosphorus and nitrogen retention of children. Effects of acid-forming and base-forming diets. Am. J. Dist. Child., vol. 49, p. 611.

HAWK, P. B., AND O. BERGEIM 1931 Practical Physiological Chemistry. 10th ed. D. Blakiston's Son and Co., Philadelphia, p. 929.

HAWKS, J. E., M. DYE AND M. M. BRAY 1937 An improved technic for metabolism studies in pre-school children with a statistical determination of its reliability. J. Nutrition, vol. 13, p. 51.

HAWKS, J. E., M. M. BRAY AND M. DYE 1938 The influence of diet on the nitrogen balances of pre-school children. J. Nutrition, vol. 15, p. 125.

HUMMEL, F. C., H. R. STERNBERGER, H. A. HUNSCHER AND I. G. MACY 1936 Metabolism of women during the reproductive cycle. VII. Utilization of inorganic elements (a continuous close study of a multipara). J. Nutrition, vol. 11, p. 235.

LONG, Z., AND M. S. PITTMAN 1935 Utilization of meat by human subjects. II. The utilization of the nitrogen and phosphorus of round and liver of beef. J. Nutrition, vol. 9, p. 677.

LUNDELL, G. E F., AND J. I. HOFFMAN 1923 Notes on the determination of phosphorus. J. Ind. and Eng. Chem., vol. 15, p. 44.

MACY, I. G., H. A. HUNSCHER, B. NIMS AND S. S. MCCOSH 1930 Metabolism of women during the reproductive cycle. I. Calcium and phosphorus utilization in pregnancy. J. Biol. Chem., vol. 86, p. 17.

MARTIN, C. J., AND R. ROBISON 1922 The minimum nitrogen expenditure of man and the biological value of various proteins for human nutrition. Biochem. J., vol. 16, p. 407.

MCCANDLESS, J. M., AND J. I. BURTON 1924 Sources of error in the determination of phosphoric acid by the molybdate-magnesia method. J. Ind. and Eng. Chem., vol. 16, p. 1267.

MEYERS, V. C., AND M. S. FINE 1919 The relative importance of the intestines and kidneys as excretory channels. Proc. Soc. Exp. Biol. and Med., vol. 16, p. 73.

NEUMANN, A. 1902–1903 Einfache Veraschung Methods (Säuregemisch-Veraschung). Z. Physiol. Chem., Bd. 37, S 115.

PORTER-LEVIN, T. 1933 Calcium and phosphorus metabolism of normal preschool children II. Successive balance studies showing the range of variation in calcium and phosphorus storage. J. Am. Diet. Assoc., vol. 9, p. 22.

PYLE, S. I., AND C. E. HUFF (WITH R. DAVIS) 1936 The use of 3-day periods in human metabolism studies. Calcium and phosphorus. J. Nutrition, vol. 11, p. 495.

SHERMAN, H. C. 1937 Chemistry of Food and Nutrition 5th ed. The Macmillan Co., New York, p. 640.

SHOHL, A. T., AND A. SATO 1923 Acid-base metabolism. II. Mineral metabolism. J. Biol. Chem., vol. 58, p. 257.

A LONG-TIME STUDY OF NITROGEN, CALCIUM AND PHOSPHORUS METABOLISM ON A MEDIUM-PROTEIN DIET [1]

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INTRODUCTION

Considerable fluctuation in nitrogen, calcium and phosphorus retentions of three young women on a constant low-protein diet supplying 0.42 gm. protein per kilogram over a 45-day period was reported in a previous paper (Kunerth and Pittman, '39). Because smaller fluctuations in nitrogen retention might be expected on a higher protein level, a diet three times as high in protein divided into fifteen 3-day experimental periods, as in the other study, was planned to determine the extent and nature of these differences.

The three women on the previous experiment and an additional subject ate a diet similar to that of the low-protein study. Subjects A and I received 40, and M and B 45 Calories per kilogram. All subjects were to receive twice their requirement of protein as recommended by Sherman ('37) and again 85% was to be furnished by meat. The calcium and phosphorus levels were not changed appreciably.

The higher protein allowance permitted a somewhat more liberal diet (table 1). Graham crackers and butter were substituted for the agar cracker and butterfat previously used and apples were added. The procedure used for preparing and serving the food, collection of samples and methods of analysis were the same as reported in the previous paper.

[1] Contribution no. 82.

TABLE 1

Daily food intakes on a medium-protein diet[1]

SUBJECT	COMPOSITION OF DIET	BEEF ROUND	BEVERAGE[2]	BUTTER	CABBAGE SALAD[3]	GRAHAM CRACKERS	APPLE	AGAR	$CaH_4(PO_4)_2 \cdot H_2O$	$Ca(C_6H_7O_8)_2 \cdot 5H_2O$	$FeC_6H_5O_7 \cdot 3H_2O$	NaCl	TOTAL
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
M	Weight	243.0	593.0	78.0	113.1	43.2	119.3	1.0	0.898	1.092	0.0316	2.000	9.85
	Nitrogen	8.63	0.33	0.12	0.16	0.57	0.04						0.409
	Calcium	0.016	0.045	0.008	0.030	0.014	0.011		0.143	0.142			0.879
	Phosphorus	0.485	0.068	0.012	0.023	0.056	0.014		0.221				3.63
	Fiber				0.79	0.65	1.19	1.00					51.40
	Ml. excess N. acid	33.79				3.37			14.24				37.41
	Ml. excess N. base		22.07		3.45		4.49			7.07	0.33		
B	Weight	254.0	611.0	78.0	113.1	47.5	119.3	1.0	0.955	1.177	0.0294	2.000	10.30
	Nitrogen	9.02	0.33	0.12	0.16	0.63	0.04						0.431
	Calcium	0.017	0.045	0.008	0.030	0.015	0.011		0.152	0.153			0.921
	Phosphorus	0.507	0.068	0.012	0.023	0.062	0.014		0.235				3.69
	Fiber				0.79	0.71	1.19	1.00					54.18
	Ml. excess N. acid	35.33				3.70			15.15				37.94
	Ml. excess N. base		22.07		3.45		4.49			7.62	0.31		
A	Weight	274.0	608.0	104.0	79.4	50.1	119.3	1.0	1.048	1.301	0.0268	2.000	11.08
	Nitrogen	9.72	0.33	0.17	0.16	0.66	0.04						0.467
	Calcium	0.018	0.045	0.010	0.030	0.016	0.011		0.167	0.170			0.986
	Phosphorus	0.546	0.068	0.012	0.023	0.065	0.014		0.258				3.73
	Fiber				0.79	0.75	1.19	1.00					58.63
	Ml. excess N. acid	38.10				3.91			16.62				38.71
	Ml. excess N. base		22.07		3.45		4.49			8.42	0.28		
I	Weight	305.0	596.0	78.0	124.1	64.8	119.3	1.0	1.211	1.561	0.0225	2.000	12.44
	Nitrogen	10.93	0.33	0.12	0.16	0.86	0.04						0.531
	Calcium	0.020	0.045	0.008	0.030	0.021	0.011		0.193	0.203			1.107
	Phosphorus	0.608	0.068	0.012	0.023	0.084	0.014		0.298				3.95
	Fiber				0.79	0.97	1.19	1.00					66.66
	Ml. excess N. acid	42.39				5.06			19.21				40.35
	Ml. excess N. base		22.07		3.45		4.49			10.11	0.23		

[1] Based on analysis of foods by periods.

[2] 150 gm. strained orange juice, 350 gm. commercial grape juice, and 50 gm. lactose for all subjects. Subject M had 43 gm. sucrose; subject B, 61 gm.; subject A, 58 gm.; and subject I, 46 gm.

[3] All subjects had 79.4 gm. shredded cabbage; subjects M, B and I had 0.7 gm. citric acid; subjects M and B each had 33.0 gm. corn oil; subject I, 44.0 gm.

RESULTS

The subjects remained in good health throughout the experiment and maintained almost constant weights, indicating that their caloric intakes met their energy requirements satisfactorily. Analysis of the foods showed that intakes of nitrogen, calcium, and phosphorus were quite constant throughout the fifteen consecutive 3-day periods.

The averages of basal metabolic tests made weekly throughout the study were —3.0, —9.5, —9.7 and —5.6% for subjects M, B, A and I, respectively (Aub-DuBois standard as modified by Boothby and Sandiford). A comparison of the basal means for the three subjects who had also served in the previous study showed that two were lower for this period and one was higher. Increasing the amount of protein approximately 300% or from 0.42 gm. per kilogram to 1.23 gm. per kilogram did not change the basal metabolic rate consistently nor to any marked degree. This agrees with the statement of Forbes et al. ('35) that increased protein to improve the nutritive value in equicaloric diets of rats "made no change in the basal heat production per unit of computed surface area."

Nitrogen findings. Analysis of the food showed that the diet supplied 194% of the protein requirement or 1.2 gm. per kilogram so that, for an adult, it may be regarded as a medium-protein diet. The meat furnished 87.7% of the nitrogen. The average daily nitrogen intakes for the four subjects, M, B, A and I were 9.85, 10.30, 11.08 and 12.44 gm. (table 2) or 197.4, 198.5, 196.1 and 194.7 mg. per kilogram, respectively, with standard deviations of 0.83, 0.77, 0.72 and 0.66 mg. The maximum variation in nitrogen intakes during the fifteen periods was 3.2 mg. per kilogram for subject M and the minimum was 2.1 for subject A.

The dry weight of the feces for the four subjects averaged 268.5 mg. per kilogram per day 37.3 mg. less than on the low-protein diet. The mean daily per capita fiber intakes of 3.9 gm. for the low and 3.8 gm. for the medium-protein study do not explain this difference. Two subjects actually increased their fiber intake slightly over the earlier study while

one subject (A) consumed 22.9% less. All subjects showed decrease in bulk of feces ranging from 6.6% for subject M to 14.0% for subject A.

Fecal nitrogen averaged 56.5 mg. per gram dry feces ranging from 41.8 to 64.3 mg. per gram for the different subjects.

TABLE 2

Average daily nitrogen, calcium and phosphorus balances on a medium-protein diet

SUBJECT	WEIGHT OF SUBJECT	AVERAGE DAILY INTAKE		AVERAGE DAILY OUTPUT			BALANCE	
		Meat	Total	Urine	Feces	Total	Total	Per kilogram
Nitrogen								
	kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*
M	49.9	8.63	9.85	7.50	0.84	8.34	+1.51	+30.3
B	51.9	9.02	10.30	7.94	0.89	8.83	+1.47	+28.3
A	56.5	9.72	11.08	8.70	0.82	9.52	+1.56	+27.6
I	63.9	10.93	12.44	9.85	0.77	10.62	+1.82	+28.5
Mean	55.6	9.58	10.92	8.50	0.83	9.33	+1.59	+28.8
Calcium								
M		0.016	0.409	0.116	0.318	0.434	−0.025	− 0.5
B		0.017	0.431	0.164	0.307	0.471	−0.040	− 0.8
A		0.018	0.467	0.128	0.378	0.506	−0.039	− 0.7
I		0.020	0.531	0.100	0.489	0.589	−0.058	− 0.9
Mean		0.018	0.460	0.127	0.373	0.500	−0.040	− 0.7
Phosphorus								
M		0.485	0.879	0.629	0.252	0.881	−0.002	± 0.0
B		0.507	0.921	0.656	0.267	0.923	−0.002	± 0.0
A		0.546	0.986	0.604	0.253	0.857	+0.129	+ 2.3
I		0.608	1.107	0.734	0.343	1.077	+0.030	+ 0.5
Mean		0.537	0.973	0.656	0.279	0.935	+0.039	+ 0.7

This approximated the mean of 54.9 mg. of the low-protein diet, but the latter was less constant. This slightly higher excretion of fecal nitrogen per gram of dry feces on the medium-protein diet was balanced by the slightly smaller amount of feces, so the mean total nitrogen excreted in the feces was almost the same for the two levels of protein, being 0.86 gm. per capita daily for the low and 0.83 gm. for the medium-protein diet. This agrees with the findings of Hawks et al.

('38) with children that a fairly constant amount of nitrogen was excreted in the feces and that it apparently had little relation to variations in intake of nitrogen.

The percentage of nitrogen intake excreted in the urine was increased from 68.3% on the low-protein to 77.8% at the medium level. That excreted in the feces was correspondingly lower amounting to 21.4 and 7.6%, respectively. The mean total nitrogen excretion representing 90.0% of the intake on the low-protein fell to 85.4% with the medium-protein intake.

Hawks' study of five children indicated that on constant diets containing 3 gm. protein per kilogram, the same proportion of the nitrogen intake was excreted. This was apparently true of the four subjects of this study, the percentage of the total nitrogen excreted being 84.7, 85.7, 85.9 and 85.4%. It was less true of the three subjects on the low-protein diet, the percentage of intake excreted ranging from 86.0 to 95.2%. This suggests that the total nitrogen excretion becomes more constant as the protein is increased in the diet.

The apparent coefficient of digestibility was increased on the medium-protein diet to a mean of 92.4% compared with 78.6% on the low-protein, a difference of 13.8%. This agrees with Murlin and Mattill ('38) that lower values in a low-protein diet may be attributed to the low level of protein in the diet, which is not favorable to a high coefficient of digestibility. Forbes et al. ('35) found increased efficiency of digestion of protein in rats by increasing the protein content of equicaloric diets. Hawks et al. ('38) reported an increase of as much as 2.6% in coefficients of digestibility for children when changed from a medium- to a high-protein diet. As protein standards for children are higher than those of adults, it must be remembered that her medium level of 3 gm. protein per kilogram was higher than that of this study of adults of 1.2 gm. per kilogram.

The mean coefficient of digestibility ranged from 91.4 to 93.8% for the various subjects although B dropped to a low

of 88.3% and I reached a high of 95.8% in one period. Subject M showed the least difference in coefficients of digestibility during the fifteen periods (4.7%) and subject I the most (6.9%). The mean nitrogen absorption was 181.5 mg. per kilogram compared with 61.0 mg. in the low-protein study.

The medium-protein diet increased the apparent coefficient of digestibility of the nitrogen by decreasing the proportion of intake excreted in the feces and increasing that in the urine. The retention was higher and the percentage of intake retained increased slightly.

Hawks et al. ('38) found change from a medium- to a high-protein diet did not increase the percentage of intake stored by children. With these adult women subjects, nitrogen retention was higher on the medium-protein diet increasing from 7.8 mg. per kilogram or 10.1% of the intake on the low-protein to 28.7 mg. per kilogram or 14.6% of the intake on the medium-protein diet. The mean nitrogen retention for all subjects was +1.59 gm. (table 2) daily, ranging from an average of +1.47 gm. for subject B to +1.82 gm. for subject A. In all of the sixty periods for the four subjects (table 3) there was but one negative nitrogen balance (0.8 mg. per kilogram per day for subject M) which may be considered equilibrium. The highest daily retention was +52.1 mg. per kilogram for subject I for period 6 although all subjects at numerous times had retentions exceeding 2 gm. daily. These unexpectedly high retentions by these young adult women continued throughout the study though the average for the total amount retained was 3.35 gm. higher in the first half of the experiment, perhaps indicating a slight tendency to decreased storage as the diet was continued. Subject I, 31 years of age, retained an average of 28.5 mg. per kilogram daily which, when compared with the mean retention of 28.7 mg. per kilogram for the entire group, showed no tendency to decrease the storage with greater maturity.

Calcium findings. The average daily calcium intakes were 0.409, 0.431, 0.467 and 0.531 gm. for M, B, A and I, respectively, or, expressed in milligrams per kilogram, 8.20, 8.30, 8.27 and 8.31 with standard deviations of 0.15, 0.14, 0.14 and 0.12 mg.

TABLE 3

Average daily nitrogen, calcium and phosphorus retentions per kilogram on a medium-protein diet

PERIOD	SUBJECT M			SUBJECT B			SUBJECT A			SUBJECT I		
	N	Ca	P	N	Ca	P	N	Ca	P	N	Ca	P
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	+33.3	−1.0	−0.3	+30.1	−1.9	±0.0	+20.5	−2.0	−0.7	+16.7	− 6.0	−3.5
2	+37.5	−1.4	+0.5	+36.6	−0.7	−0.1	+29.9	+0.8	+1.3	+26.6	− 3.9	−0.9
3	+13.9	−1.4	−1.5	+19.4	−2.0	−0.1	+40.4	−0.2	+1.0	+29.4	− 1.0	+1.0
4	+20.5	−0.4	−2.0	+22.9	−0.4	−0.1	+29.9	+0.8	−0.2	+30.2	− 1.8	±0.0
5	+30.6	+0.1	+0.1	+20.3	−1.3	−1.6	+23.9	−0.7	+2.6	+34.6	+ 0.3	+1.0
6	+36.0	+0.6	−0.4	+28.6	−0.4	−0.7	+22.5	−0.7	+3.3	+52.1	+ 1.1	+1.9
7	+47.3	+1.2	+2.1	+36.5	+0.4	+0.8	+39.8	−2.3	+2.9	+29.0	+ 0.3	+0.7
8	+29.6	−1.0	−0.3	+31.5	−0.7	−0.1	+32.2	−0.1	+3.0	+25.2	− 1.9	+0.2
9	+31.8	+1.0	+1.7	+32.0	−0.3	−0.9	+16.6	−1.5	−0.6	+24.9	− 0.6	+0.6
10	+39.3	+0.4	+2.8	+34.2	+0.7	+1.2	+19.1	+0.5	+5.0	+12.1	− 0.6	+1.1
11	+17.2	−0.8	+0.4	+36.4	−1.0	+0.4	+29.4	−0.9	+2.2	+39.3	− 2.3	+2.1
12	+37.5	+0.3	+0.8	+41.0	+0.2	+1.9	+37.5	−0.3	+5.1	+19.1	− 2.4	−2.0
13	− 0.8	−1.9	−3.3	+32.5	+1.4	+2.6	+29.7	−1.3	+4.0	+12.4	± 0.0	−0.1
14	+39.1	−1.6	−0.7	+15.7	−2.2	−1.5	+22.1	+1.5	+3.0	+39.6	+ 2.4	+2.1
15	+42.3	−1.4	−0.5	+ 6.1	−3.2	−2.4	+21.2	−3.6	+3.2	+36.8	+ 2.4	+2.7
Mean	+30.3	−0.5	±0.0	+28.3	−0.8	±0.0	+27.6	−0.7	+2.3	+28.5	− 0.9	+0.5
						Per cent of intake						
Mean	15.3	−6.1	0.0	14.3	−9.6	0.0	14.1	−8.5	13.1	14.6	−10.8	2.9

The largest variation in calcium intake during the fifteen periods, 0.52 mg. per kilogram, occurred in the case of subject M. Subject I had the least variation amounting to 0.42 mg. per kilogram.

The average calcium output for all subjects was 0.500 gm. per day or 9.0 mg. per kilogram which resulted in an average retention of —0.7 mg. per kilogram. The average daily fecal calcium for all subjects was 6.7 and the urinary calcium was 2.3 mg. per kilogram but the amounts varied considerably throughout the study. For the fifteen periods subjects M, B, A and I excreted 73, 65, 75 and 83% of the total calcium output in the feces.

Phosphorus findings. Subjects M, B, A and I received daily 0.879, 0.921, 0.986 and 1.107 gm. of phosphorus or 17.6, 17.7, 17.5 and 17.3 mg. per kilogram with standard deviations of 0.17, 0.14, 0.13 and 0.13 mg. A maximum variation of 0.65 mg. per kilogram for subject M and a minimum of 0.46 mg. for subject I occurred in phosphorus intakes.

The subjects excreted 0.881, 0.923, 0.857 and 1.077 gm. per day, indicating that over the 45-day period subjects M and B were in equilibrium and subjects A and I were storing phosphorus, particularly A. The average fecal phosphorus amounted to 28.6% of the intake and ranged from a low of 4.5 mg. per kilogram for subject A to a high of 5.4 mg. per kilogram for subject I. Of the total output, 71% was urinary phosphorus for subjects M, B and A, whereas for I, it was 68%.

In the previous experiment in which three of these subjects had a moderately low protein diet but received 6.8% more calcium and 4.3% more phosphorus, all had average negative calcium balances for the study and two had negative phosphorus balances. In the present study these same subjects had less negative calcium balances (6.4, 12.4 and 9.9%, respectively) and their phosphorus retentions improved in two cases and in the third remained at approximate equilibrium. A comparison of the urinary and fecal calcium outputs of the three subjects serving on both diets showed that the

smaller negative balances on the higher protein diet were due to smaller fecal loss, as the urinary calcium was about the same for both diets. To a somewhat less extent, the same is true for phosphorus.

The improved calcium retention can scarcely be explained on the basis of the difference in the excess acid of the two diets as the former had about twice as much excess acid as the higher protein diet. Davis ('35) in comparing retention and path of excretion of nitrogen, calcium and phosphorus of children on acid-and-base forming diets found greatest effect on calcium. However, her results indicate that on a basic diet the proportion of fecal calcium was increased and urinary calcium decreased but retention was not influenced.

The fiber content of the two diets differed little except for subject A, but its effect on the mineral utilization is doubtful. In the low-protein diet subject A had more fiber in the diet than the other subjects and excreted more fecal calcium but less phosphorus than the others. In the present study she had about the same amount of fiber as the other subjects, about a fourth less than she had had on the previous diet, but again she had higher fecal calcium and lower phosphorus than subjects M and B. Ascham ('30–'31) found some evidence that fecal calcium in dogs was increased by increased amounts of agar but discrepancies occurred. Some attention has been given to mineral utilization on protein enriched diets in other studies. Conner and Sherman ('36) found, with increase of protein in the diet from 14.0 to 18.8%, a gain in body calcium in 2- to 3-month-old rats receiving minimum adequate calcium allowances. However, Woodman et al. ('37) in a study of the nutrition of the bacon pig found no differences in mineral retention that might be correlated with an increase from a normal to a high level of protein intake.

A total of six menstrual periods occurred during the study: two each for subjects M and A, and one each for subjects B and I. Retentions were above the average for the study during the menstrual periods in all six cases for nitrogen, in five for calcium, and in four for phosphorus. No particularly unfavorable effects on retentions could be noted at these times.

The higher intake of protein gave somewhat better retentions than were noted on the low-protein diet of the earlier study. No menstrual collections were made. What changes analyses of the menstrua would have made cannot be predicted.

CONCLUSIONS

1. A medium-protein diet did not eradicate the tendency to variation in retention of nitrogen, calcium and phosphorus observed in the low-protein diet of a previous study.

2. A constant diet furnishing 1.2 gm. protein (87.7% from beef) and 40 to 45 Calories per kilogram enabled four young adult women to maintain a positive balance for nitrogen and phosphorus during forty-five consecutive balance days divided into fifteen periods of 3 days each. No subjects were in positive calcium balance, though improved retention over the low-protein study was noted.

3. The medium-protein diet used in this study improved appreciably the utilization of nitrogen, calcium and phosphorus by human subjects compared with the low-protein diet of the earlier investigation.

With this increase in protein the level used for calcium was inadequate to maintain these subjects under the conditions of this study in equilibrium during a 45-day period.

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LITERATURE CITED

ABCHAM, L. 1930–1931 The influence of bulk in the diet upon fecal calcium and phosphorus. J. Nutrition, vol. 3, p. 411.

CONNER, R. T., AND H. C. SHERMAN 1936 Some aspects of protein intake in relation to growth and rate of calcification. J. Biol. Chem., vol. 115, p. 695.

DAVIS, N. J. 1935 Calcium, phosphorus and nitrogen retention of children. Effects of acid-forming and base-forming diets. Am. J. Dis. Child., vol. 49, p. 611.

FORBES, E. B., R. W. SWIFT, A. BLOCK AND O. J. KAHLENBERG 1935 The utilization of energy producing nutriment and protein as affected by individual nutrient deficiencies. III. The effects of the plane of protein intake. J. Nutrition, vol. 10, p. 461.

HAWKS, J. E., M. M. BRAY AND M. DYE 1938 The influence of diet on the nitrogen balances of pre-school children. J. Nutrition, vol. 15, p. 125.

KUNERTH, B. L., AND M. S. PITTMAN 1939 A long-time study of nitrogen, calcium and phosphorus metabolism on a low-protein diet. J. Nutrition, vol. 17, p. 161.

MURLIN, J. R., AND H. A. MATTILL 1938 Digestibility and nutritional value of cereal proteins in the human subject. J. Nutrition, vol. 16, p. 15.

SHERMAN, H. C. 1937 Chemistry of Food and Nutrition. 5th ed. The Macmillan Co., New York, p. 640.

WOODMAN, H. E., R. E. EVANS AND W. G. TURPITT 1937 The nutrition of the bacon pig. II. The influence of high-protein intake on protein and mineral metabolism. J. Agr. Sci., vol. 27, p. 569.

A STUDY OF BREED AND SEASONAL VARIATIONS IN THE ASCORBIC ACID CONTENT OF CERTIFIED MILK FROM GUERNSEYS AND HOLSTEINS

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ONE FIGURE

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The ascorbic acid content of cow's milk has been studied by many investigators. Barnes and Hume ('19) found a seasonal variation in the antiscorbutic properties of cow's milk. In 1920 Dutcher, Eckles, Dahle, Mead and Schaefer reported that the vitamin content of cow's milk is dependent upon the vitamin content of the ration ingested by the cow. Hart, Steenbock and Ellis ('20) concluded that summer pasture milk is much richer in the antiscorbutic vitamin than dry feed milk or winter milk, produced with a ration which included corn silage or sugar mangels. Hess, Unger and Supplee ('20) assayed dried milks and found that the milk from cows on pasture serves as a fair source of vitamin C whereas the milk from cows receiving a feed low in vitamin C possessed practically no antiscorbutic properties.

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Olson and Copeland ('24) stated that the vitamin C in milk has its origin in the feed of the cow, and that good silage is important in order to maintain an adequate supply of this vitamin in the milk.

MacLeod ('27) reported that "the greater antiscorbutic value of the milk of the pasture-fed cow is doubtless due to the antiscorbutic vitamin content of the fresh grasses which the animal receives, since the animal body probably does not synthesize this vitamin and is therefore dependent on its food to provide an adequate amount of it in its milk."

Rasmussen, Guerrant, Shaw, Welch and Bechdel ('36) stated that the ascorbic acid content of milk is apparently dependent solely upon the ascorbic acid content of the diet after the first 2 months of lactation. Hannisdahl ('37) reported that the ascorbic acid content of the milk from stall-fed cows was lower than that from pasture-fed cows.

On the other hand, Hughes, Fitch, Cave and Riddell ('27) concluded from their experiments "that the vitamin C content of a cow's ration has little if any influence on the vitamin C content of its milk." These investigators also stated the dairy cow can synthesize vitamin C to some extent.

In 1936 Riddell, Whitnah, Hughes and Lienhardt, in a study concerning the influence of rations on the vitamin C content of milk found that the rations studied had no significant influence on the vitamin C content of cow's milk. Whitnah and Riddell ('37) fed three cows approximately 65 kg. per day of green rye which contained 0.86 mg. of vitamin C per gram and found that "15 days after the initial heavy rye feeding there was no significant increase in the vitamin C concentration of the milk from any of the three cows." Reedman ('37) reported that dairy cattle apparently have a mechanism for synthesizing ascorbic acid and therefore are not susceptible to vitamin C deficiency. West and Wenger ('38) found no apparent difference in the ascorbic acid content of the cow's milk after the addition of orange meal to the feed.

Unfortunately, the majority of the conclusions cited above are based upon the results obtained from studies of only a

few days to a few months in duration. Furthermore in some cases the data have been gathered from experiments which included only two or three cows. In view of the short experimental periods, the small number of cows employed in most of the experiments and the lack of agreement in the published results, it seemed desirable to conduct a study of the vitamin C content of certified milk, produced under controlled conditions, by a group of twenty-five to thirty cows during a complete year.

Twenty-eight typical Holstein and Guernsey cows were selected for this study from a herd of 200 cows maintained under carefully controlled conditions for the production of certified milk. The experimental cows were housed with the remainder of the herd in modern dairy barns throughout the period of the experiment. They were continuously stall-fed and at no time had access to pasture. To guard against disease the cows were under the direct supervision of an experienced veterinarian, the attendants were under constant medical inspection, and before a cow was added to the herd she was subjected to a 60-day quarantine in an isolation barn in order to eliminate any animal affected with mastitis, infectious abortion or other contagious bovine diseases.

From time to time it was necessary to remove cows from the study on account of diminished milk flow. These were replaced by representative cows selected from the certified milk herd. When the cows that had been removed from the study again freshened they were returned to the experiment. Hence at all times during this investigation the experimental animals consisted of cows which represented practically all stages of lactation. As a result of replacing cows during non-lactating periods, fifty-five different cows were used in this study. Accordingly the results obtained should be fairly applicable to certified milk produced by the best managed commercial dairies.

Obviously data concerning the age, size, physical condition and number of pregnancies of the cows used are of interest for evaluating the results of this study. The age of the Guernsey cows varied from 4 to 11 years and that of the

Holstein cows from 5 to 9 years. The weight of the Guernsey cows varied from 840 pounds to 1170 pounds whereas the Holstein cows varied from 885 pounds to 1300 pounds. The average weight of the Holstein cows (1088 pounds) was materially greater than that of the Guernsey cows (972 pounds). All of the cows, except three or four Holsteins, were in very satisfactory physical condition. Seven cows were in their second lactation period and three were in their sixth lactation period but the great majority were in their third or fourth lactation periods.

All cows received a daily ration compounded from various combinations of the following ingredients: first and second grades of alfalfa hay, first and second grades of mixed hay, beet pulp, dairy ration, dairy ration with 6.7% irradiated yeast, home mixed fitting ration with 6.7% irradiated yeast and a commercial fitting ration with irradiated yeast. The composition of the dairy and fitting ration is reported in table 1. Samples of these products were assayed for their ascorbic acid content by Tillman's method as modified by Bessey and King ('33). Five gram samples were ground with 30 gm. of acid-washed sand; 25 to 35 cc. of an acid mixture containing 4% metaphosphoric and 8% acetic acid was added as suggested by Musulin and King ('36). The mass was centrifuged, the supernatant liquid was decanted and the residue repeatedly extracted until 50 cc. of extract were obtained. Five cubic centimeters of the extract, diluted to 25 cc. were titrated with 2,6-dichlorophenolindophenol as described by Tripp, Satterfield and Holmes ('37). The results of these assays are reported in table 2.

These values are less than those of Reedman ('37) who reported that pasture grass contained 20.5 to 136.0 mg. total ascorbic acid per 100 gm. He also mentioned that hay may contain as much as 30 mg. ascorbic acid per 100 gm., whereas in the case of ensilage 12% to 50% of the original ascorbic acid may be preserved for a period of 6 months depending upon the method of preservation. Reedman estimates that a dairy cow on winter ration receives approximately 9 gm. of ascorbic acid daily in the ration.

TABLE 1

Composition of dairy and fitting rations

Dairy ration

	%
Wheat bran	25.0
Oats (crimped)	17.5
Yellow hominy feed	10.0
Distillers' corn grains dried	10.0
Corn gluten feed	10.0
Cottonseed meal, 41% (hydrolic process 5% fat)	10.0
Yellow cornmeal	5.0
Soy bean oil meal (expeller process)	5.0
Cane molasses	5.0
Iodized salt	1.0
Steamed bone meal	1.0
Calcium carbonate	0.5

Home mixed fitting ration

Oats (crimped)	30.0
Wheat bran	25.0
Yellow hominy feed	15.0
Yellow cornmeal	10.0
Soy bean oil meal (expeller process)	10.0
Cane molasses	7.0
Steamed bone meal	1.0
Calcium carbonate	1.0
Iodized salt	1.0

Commercial fitting ration

Cornmeal	Unknown
Corn gluten meal	Unknown
Crushed oats	Unknown
Soy bean oil meal	Unknown
Wheat middlings	Unknown
Wheat bran	Unknown
Alfalfa meal	Unknown
Dried beet pulp	Unknown
Molasses	Unknown
Calcium carbonate	Unknown
Iodized salt	Unknown
Carotene	Unknown

EXPERIMENTAL

Samples of morning milk were taken from each of the twenty-eight cows at bi-monthly intervals during the entire experimental year. The representative samples were placed in 1 ounce flint glass bottles which were immediately placed on ice and protected from light. The milk samples were tested by the indophenol titration method employing 2,6-dichlorophenolindophenol. The vitamin C was determined by the method of King ('37). Five cubic centimeters of milk were

TABLE 2

The ascorbic acid content of the components of the experimental ration

	Ascorbic acid per 100 gm. of feed mg.
Good farm hay (one-half alfalfa)	1.334
Second grade farm hay (all grass)	0.907
Grass ensilage	1.548
First grade baled alfalfa	1.883
Second grade baled alfalfa	1.380
Beet pulp	0.669
Dairy ration	0.562
Dairy ration with irradiated yeast	0.754
Fitting ration and irradiated yeast	0.592
Commercial fitting ration with irradiated yeast	1.622

added to a 2.5 cc. mixture of 8% acetic acid and 4% metaphosphoric acid in a 50 cc. Erlenmeyer flask. The dye solution was added from a microburette drop by drop until a faint pink end point was reached which remained for 30 seconds. The strength of the dye solution was equivalent to 0.08 to 0.10 mg. of vitamin C per cubic centimeter. This solution was prepared and standardized in a manner similar to that described by Tripp, Satterfield and Holmes ('37).

RESULTS

The results of the bimonthly assays of certified milk are reported in table 3 which shows the number of cows producing milk, the minimum, maximum and average ascorbic acid content of the Guernsey and Holstein milks at each assay

period. The number of Guernsey cows under observation varied from 10 to 15 but usually there were thirteen or fourteen, and the number of Holsteins varied from eleven to sixteen with an average of fourteen. All the values for ascorbic acid refer to the milligrams per 1000 cc. of milk. The minimum ascorbic acid content of Guernsey milk varied from

TABLE 3

Ascorbic acid content of certified cow's milk (milligrams per 1000 cc. milk)

GUERNSEYS					HOLSTEINS				
Date	Number of cows	Minimum	Maximum	Average	Date	Number of cows	Minimum	Maximum	Average
Aug. 19	11	15.81	24.63	18.93	Aug. 19	11	14.34	21.32	17.55
Sept. 16	14	17.24	29.81	20.26	Sept. 16	14	15.45	21.55	18.05
Sept. 30	14	12.32	25.80	18.63	Sept. 30	14	15.02	22.33	18.95
Oct. 14	13	19.21	31.37	21.75	Oct. 14	12	14.51	23.13	19.46
Oct. 28	14	16.74	27.50	21.29	Oct. 28	13	16.73	25.27	20.44
Nov. 10	14	18.69	25.70	21.96	Nov. 10	14	16.35	24.76	20.02
Nov. 24	14	16.95	27.03	21.41	Nov. 24	14	15.95	21.82	19.48
Dec. 9	15	11.07	27.68	20.86	Dec. 9	14	15.30	23.44	19.72
Dec. 23	14	16.24	22.74	19.38	Dec. 23	14	12.35	22.42	17.18
Jan. 6	14	18.52	23.72	21.26	Jan. 6	14	13.97	20.47	18.24
Jan. 20	14	18.98	27.00	22.59	Jan. 20	14	13.50	22.99	19.62
Feb. 3	14	18.63	26.00	21.92	Feb. 3	14	14.05	23.54	19.53
Feb. 17	14	19.10	28.23	23.37	Feb. 17	15	15.78	24.91	20.23
Mar. 3	14	18.30	25.16	21.05	Mar. 3	15	13.72	20.97	18.22
Mar. 23	14	14.95	23.77	20.31	Mar. 23	15	11.50	19.94	16.89
Apr. 7	14	15.22	23.02	19.22	Apr. 7	14	11.88	19.68	16.82
Apr. 21	14	13.78	22.30	19.35	Apr. 21	14	9.84	19.35	15.72
May 6	14	13.97	21.66	17.76	May 6	15	11.52	18.15	16.04
May 19	13	17.02	24.03	20.36	May 19	16	11.68	21.36	17.82
June 2	13	17.61	22.09	19.47	June 2	15	10.70	19.33	16.89
June 23	13	17.83	23.65	20.12	June 23	15	10.97	19.88	16.55
July 8	13	15.85	22.93	20.30	July 8	15	14.84	21.60	17.70
July 21	13	15.50	22.14	18.45	July 21	14	14.62	19.50	16.99
Aug. 11	10	19.31	25.48	22.84	Aug. 11	12	14.05	21.08	17.89
Average	13	16.62	25.14	20.54	Average	14	13.69	21.62	18.17

11.07 mg. on December 9th to 19.31 mg. on August 11th, and the maximum varied from 21.66 mg. on May 6th to 31.37 mg. on October 14th with averages varying from 17.76 mg. on May 6th to 23.37 mg. on February 17th. The minimum ascorbic acid content of Holstein milk varied from 9.84 mg. on April 21st to 16.73 mg. on October 28th; the maximum varied

from 18.15 mg. on May 6th to 25.27 mg. on October 28th with averages varying from 15.72 mg. on April 21st to 20.44 mg. on October 28th.

The average ascorbic acid content of the 324 samples of Guernsey milk assayed during the year's experiment was 20.54 mg. and that of the 337 samples of Holstein milk was 18.17 mg. These data are in agreement with those recently published by West and Wenger ('38) who found that Guernsey milk was superior to that from the Holstein cow as

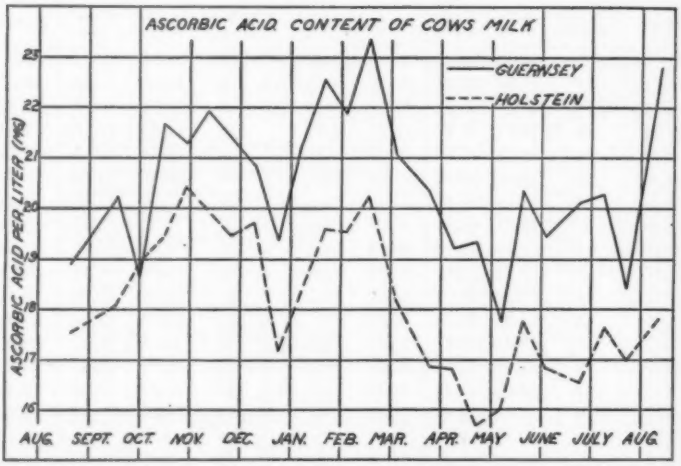

Fig. 1 The ascorbic acid content of certified milk as influenced by breeds and seasons.

a source of vitamin C. Rasmussen ('36) also found that milk from Holstein cows was inferior to that produced by Brown Swiss, Ayrshire, Guernsey and Jersey cows as sources of vitamin C.

The seasonal variations in certified milk produced by stall-fed cows maintained under conditions which were uniform for the entire year are shown by the graph on figure 1. An examination of figure 1 shows that the amount of ascorbic acid found in milk of both the Guernsey and Holstein cows tended

to be greater during October, January and February than during the other periods of the year, although there was a pronounced increase in vitamin C content of the Guernsey milk on the last day of the experiment. From the middle of December until the middle of February there appeared to be a steady and consistent increase in the ascorbic acid content of the milk of both breeds. From the middle of February until early May there was a gradual and persistent drop in the amount of ascorbic acid in both the Guernsey and Holstein milks. However, the composition of the ration was not altered previous to or during this period. The amount of vitamin C in the milk of both breeds was at its lowest point in early May. Seasonal variations in cows' milk have been observed by a number of investigators. Rohmer, Bezssonoff, Sanders and E. Stoerr ('34), in a study involving cow's milk from three different sources, found that the vitamin C content was high in November, dropped sharply in December and rose in March and April. Sharp ('36) reported that the ascorbic acid content of fresh milk is relatively constant throughout the year. Whitnah and Riddell ('37) found that the ascorbic acid content for a herd of cows increased from October to December, decreased from December to February and increased again from February to March.

On the other hand some investigators have obtained contrary results. Kon and Watson ('37) stated that the level of vitamin C in mixed milk is very constant and is independent of the season of the year. MacLeod ('27) concluded "that the milk of stall-fed cows which are given a well balanced, uniform diet throughout the year will not show seasonal variations in antiscorbutic value."

It is interesting to note in connection with the seasonal variation in the ascorbic acid content of cow's milk observed in this study that Sinkko ('37) also observed a seasonal variation in the ascorbic acid content of human milk collected from ten women at intervals throughout a year. In the present study the certified milk collected in February and early March contained the largest amount of ascorbic acid,

whereas Sinkko found the human milk to have the highest vitamin C content in August and September. Incidentally Sinkko observed that the incidence of still-births and infant mortality were lowest at the season of the year when the mothers were less likely to be vitamin C deficient.

The amounts of ascorbic acid in Guernsey and Holstein milks (20.54 and 18.17 mg. respectively) reported in this paper are greater than those observed by West and Wenger ('38). These workers concluded that raw certified milk can contribute materially to the vitamin C content of the human dietary, particularly since they observed only a 17% loss in the ascorbic acid content 34 hours after milking.

SUMMARY

Six hundred and sixty-one samples of certified milk collected at bi-monthly intervals from fifty-five Guernsey and Holstein cows over a 52-week period were assayed for their ascorbic acid content by the dichlorophenolindophenol titration method.

The ascorbic acid content of both Guernsey and Holstein milk showed seasonal variation. The February milk contained large amounts of ascorbic acid, 23.37 mg. for the Guernsey and 20.23 mg. per liter for the Holstein milk. A second peak occurred in early November when the ascorbic acid content of the Guernsey milk was 21.96 mg. and late October when the ascorbic acid content of the Holstein milk was 20.44 mg.

The average ascorbic acid content of the Guernsey milk varied from 17.76 mg. to 23.37 mg. and for the Holstein milk from 15.72 mg. to 20.44 mg. per liter. The ascorbic acid content was consistently higher for the Guernsey than for the Holstein milk. The total yearly average for the Guernsey milk was 20.54 mg. of ascorbic acid whereas that of the Holstein milk was 18.17 mg. per liter.

The data obtained in this investigation indicate that the variation in the ascorbic acid content of raw certified milk produced under commercial conditions is independent of

ration and period of lactation. However, it is evident from the above results that the generous consumption of raw certified milk can contribute materially to the vitamin C content of the human dietary.

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LITERATURE CITED

BARNES, R. E., AND E. M. HUME 1919 A comparison between the antiscorbutic properties of fresh, heated and dried cow milk. Lancet, vol. II, p. 323.

BESSEY, O. A., AND C. G. KING 1933 The distribution of vitamin C in plant and animal tissues, and its determination. J. Biol. Chem., vol. 103, p. 687.

DUTCHER, R. A., C. H. ECKLES, C. D. DAHLE, S. W. MEAD AND O. G. SCHAEFFER 1920 The influence of diet of the cow upon the nutritive and antiscorbutic properties of cow's milk. J. Biol. Chem., vol. 45, p. 119.

HANNISDAHL, B. 1937 Cow's milk as a source of vitamin C. Tidsskr. Norske Laegeforening, vol. 57, p. 1068 (Nutr. Abs. and Revs., vol. 8, p. 89, 1938).

HART, E. B., H. STEENBOCK AND N. R. ELLIS 1920 Influence of diet on the antiscorbutic potency of milk. J. Biol. Chem., vol. 42, p. 383.

HESS, A. F., L. J. UNGER AND G. C. SUPPLEE 1920 Relation of fodder to the antiscorbutic potency and salt content of milk. J. Biol. Chem., vol. 45, p. 229.

HUGHES, J. S., J. B. FITCH, H. W. CAVE AND W. H. RIDDELL 1927 Relation between the vitamin C content of a cow's ration and the vitamin C content of its milk. J. Biol. Chem., vol. 71, p. 309.

KING, C. G. 1937 Personal communication.

KON, S. K., AND M. B. WATSON 1937 The vitamin C content of cow's milk. Biochem. J., vol. 31, p. 223.

MACLEOD, F. L. 1927 Antiscorbutic vitamin content of milk of stall-fed cows through a year. Probability that ensilage is an important source of this vitamin. J. Am. Med. Assoc., vol. 88, p. 1947.

MUSULIN, R. R., AND C. G. KING 1936 Metaphosphoric acid in the extraction and titration of vitamin C. J. Biol. Chem., vol. 116, p. 409.

OLSON, T. M., AND L. COPELAND 1924 The influence of pasteurization and diet of the cow on the antiscorbutic potency of the milk. J. Dairy Sci., vol. 7, p. 370.

RASMUSSEN, R., N. B. GUERRANT, A. O. SHAW, R. C. WELCH AND S. I. BECHDEL 1936 The effects of breed characteristics and stages of lactation on the vitamin C (ascorbic acid) content of cow's milk. J. Nutrition, vol. 11, p. 425.

REEDMAN, E. J. 1937 The ascorbic acid content of milk. Canadian Pub. Health J., vol. 28, p. 339.

ROHMER, P., N. BEZSSONOFF, U. SANDERS AND E. STOERR 1934 Variation saisonniere de la teneur du lait en vitamin C. Extrait du volume des discussions des rapports et communications du VIII[e] Congres de l'Association Francaise de Pediatrie, Paris, 1934.

RIDDELL, W. H., C. H. WHITNAH, J. S. HUGHES AND H. F. LIENHARDT 1936 Influence of the ration on the vitamin C content of milk. J. Nutrition, vol. 11, p. 47.

SHARP, P. F. 1936 Vitamin C in pasteurized milk. Science, vol. 84, p. 461.

SINKKO, E. I. 1937 Relation between infantile mortality, stillbirth and variations in the vitamin C content of the mother's milk at various seasons of the year in Finland. Acta paediat., vol. 21, p. 407. (Abs. in Nutri. Abs. and Revs., 1938, vol. 8, p. 201.)

TRIPP, F., G. H. SATTERFIELD AND A. D. HOLMES 1937 Varietal differences in the vitamin C (ascorbic acid) content of tomatoes. J. Home Economics, vol. 29, p. 258.

WEST, F. V., AND J. C. WENGER 1938 The vitamin C content of certified milk at the time of consumption. Am. J. Digest. Dis., vol. 5, p. 251.

WHITNAH, C. H., AND W. H. RIDDELL 1937 Variations in the vitamin C content of milk. J. Dairy Sci., vol. 20, p. 9.